GLUCAGON-LIKE PEPTIDE-1 DELIVERY FOR TREATMENT OF TYPE 2 DIABETES

by

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SUPERVISORY COMMITTEE APPROVAL

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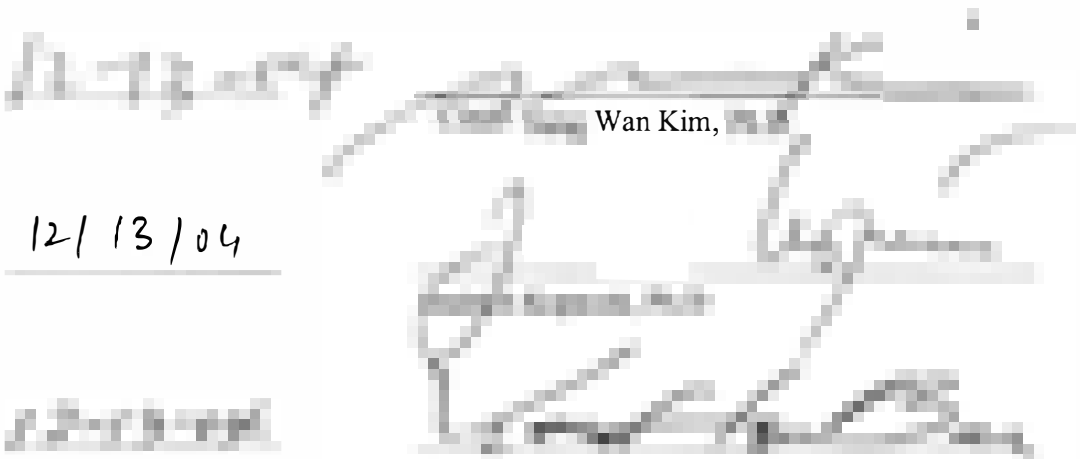

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ABSTRACT

Glucagon-like peptide-1 (GLP-1) is an insulinotropic hormone that increases insulin secretion in a glucose-dependent manner. Insulin secretion is only increased when concentrations of glucose is high and not at low to normal concentrations of glucose, therefore avoiding hypoglycemic episodes. In spite of its many remarkable advantages as a therapeutic agent for diabetes, GLP-1 is not immediately clinically applicable because of its extremely short half-life. The purpose of this research was to design an effective way of GLP-1 delivery for therapeutic use of GLP-1. The first half of the research studied the effects of GLP-1 delivery using a thermosensitive biodegradable triblock copolymer. The second half of this research was to design a new and effective GLP-1 delivery system for therapeutic use for type 2 diabetes treatment based upon construction of GLP-1 plasmid and its delivery.

The effect of GLP-1 delivery system using biodegradable injectable polymer was studied. The results from the *in vitro* release experiment did show steady amount of GLP-1 released from PLGA-PEG-PLGA triblock copolymer formulation with zinc-complexed GLP-1 depot more than ten days after loading. As a result from the animal experiment with the type 2 diabetic model, it was evident that the GLP-1 released from the thermosensitive biodegradable hydrogel formulation was bioactive as it stimulated insulin secretion *in vivo*.

Therefore, it was concluded that it is feasible to use PLGA-PEG-PLGA triblock copolymer formulation with zinc-complexed GLP-1 as a 2 week delivery system, making it a twice-a-month injection depot.

For less frequent and more convenient administration of GLP-1, the GLP-1 gene delivery system was attempted in this study. Since no reactive hypoglycemia occurs even with higher concentrations of GLP-1, a potent promoter/enhancer was used in this system. It has been established that GLP-1 gene delivery significantly decreased blood glucose levels in a type 2 diabetic animal model. The results showed that delivered GLP-1 gene stimulated insulin secretion, indicating that GLP-1 gene delivery also represents a promising candidate for treatment of type 2 diabetes.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

CMV	cytomegalovirus
DIO	diet-induced obesity
DPP-IV	dipeptidyl peptidase-IV
ELISA	enzyme linked immunosorbent assay
GIP	glucose-dependent insulinotropic polypeptide
GLP-1	glucagon-like peptide 1
IPGTT	intraperitoneal glucose tolerance test
LCST	lower critical solution temperature
NLS	nuclear localization signal
NPC	nuclear pore complex
PEG	poly(ethylene glycol)
PEI	polyethylenimine
PLGA	poly(lactide-co-glicolide)
PLL	poly(L-lysine)
RHD	Rel homology domain
RIA	radioimmunoassay
ROS	reactive oxygen species
RP-HPLC	reverse-phase high performance liquid chromatography
SV40	simian virus 40
TFA	trifluoro acetic acid
TNF	tumor necrosis factor
WSLP	water-soluble lipopolymer
ZDF	Zucker diabetgic fatty

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CHAPTER 1

INTRODUCTION

1.1 Overview of Diabetes Mellitus

1.1.1 The Impact of Diabetes in United States

There are 18.2 million people (6.3 % of the population) in the United States who have diabetes. Each day, approximately 2,200 people are diagnosed with diabetes [1]. Each year, 1.3 million people aged 20 years or older are diagnosed with diabetes [2]. Diabetes was the sixth leading cause of death listed on U.S. death certificates in 2000. This is based on the 69,301 death certificates in which diabetes was listed as the underlying cause of death [2]. Many people first become aware that they have diabetes when they develop one of its life-threatening complications, such as kidney disease, nerve disease, need for amputations, heart disease, stroke, or blindness [3]. Overall, the risk for death among diabetic patients is about two times that of people without diabetes [2].

Diabetes is one of the most costly health problems in America. The total annual economic cost of diabetes in 2000 was estimated to be $132 billion [2]. The direct costs associated with diabetes represent 5.8% of the total personal health-care expenditures in the United States; however, diagnosed diabetes patients account for only 3.8% of the total United States population [4].

1.1.2 Classification of Diabetes

Diabetes is a generalized term describing a disease that can be divided into different types. The two main types of diabetes are type 1 diabetes and type 2 diabetes. Type 1 diabetes is formerly called insulin-dependent or juvenile onset diabetes. Type 1 diabetes accounts for 5-10% of all cases of diabetes in the United States [2]. In a person with type 1 diabetes, the pancreatic beta cells are destroyed by autoimmune processes, so no insulin is produced in the pancreas [5]. Therefore, people with type 1 diabetes must take insulin for survival. Type 1 diabetes is associated with autoimmune, genetic, and environmental factors [2]. Type 2 diabetes is formerly called non-insulin-dependent or adult-onset diabetes. It arises because of insulin resistance, in which the body can not use insulin properly, combined with relative insulin deficiency. It typically presents in middle and late life, although an increasing number of children and teenagers around the world are developing it due to increase in obesity. Type 2 diabetes does not exhibit a clear set of symptoms. When it is fully developed, there will be abnormalities in both insulin secretion and insulin action. It is characterized by an increase in basal glucose concentrations, upon which are superimposed exaggerated postprandial glucose excursions, induced by a combination of β-cell dysfunction and impaired insulin sensitivity. Type 2 diabetes mellitus occurs when the pancreatic beta cell fails to generate an adequate insulin level to maintain euglycemia in the setting of insulin resistance. The molecular basis of both insulin resistance and of beta cell failure in type 2 diabetes remains unclear. Type 2 diabetes constitutes 90 – 95 % of all cases of diabetes [2], and the

incidence of type 2 diabetes is rising rapidly worldwide [6]. Risk factors for type 2 diabetes include family history of diabetes, obesity, older age, physical inactivity, prior history of gestational diabetes, impaired glucose tolerance, and ethnicity. Asian Americans, Latino Americans, Native Americans, and some Pacific Islanders are known to have particularly high risk for type 2 diabetes [2].

Gestational diabetes is diagnosed in some women during pregnancy. It is a form of glucose intolerance, and more frequently occurs among obese women and women with a family history of diabetes. Some studies have shown that 20 to 50% of women with a history of gestational diabetes develop diabetes in the next 5-10 years [2].

Other types of diabetes may account for up to 5% of all cases of diabetes. The causes of other specific types of diabetes include genetic conditions, surgery, drugs, infections, malnutrition, and other illnesses.

1.1.3 Complications of Diabetes

Diabetes is a group of clinical disorders characterized by the elevated level of blood glucose. Hyperglycemia plays an important role in the pathogenesis of all major complications associated with diabetes such as nephropathy [7], retinopathy [8], neuropathy [9], macrovascular disease [10], impaired cellular immunity [11], and abnormalities in cell growth [12] and differentiation [13].

Heart disease is the leading cause of diabetes-related deaths. The risk for heart disease and stroke is two to four times higher among people with

diabetes. More than 70% of adults with diabetes have high blood pressure which is defined as greater than 130/80 millimeters of mercury [2]. According to the American Diabetes Association, diabetes-related complications include heart disease, blindness, kidney disease, nervous system disease, dental disease, and amputations. Diabetes is the leading cause of blindness and treated end-stage renal disease. Each year, diabetic retinopathy causes from 12,000 to 24,000 new cases of blindness. A total of 41,046 people with diabetes began treatment for end-stage renal disease in 2000. About 60% to 70% of people with diabetes have nervous system damage, and more than 60% of nontraumatic lower limb-amputations in United States occur among people with diabetes. Almost one-third of people with diabetes have severe periodontal disease. People with diabetes are more susceptible to other illnesses. Furthermore, uncontrolled diabetes often leads to diabetic ketoacidosis and nonketotic coma [2].

1.1.4 Current Treatments

Although there is no known cure for diabetes, researchers have found new and improved ways of treating diabetes. In most patients with this disease, the treatment goal should be to achieve blood glucose levels as near normal as possible. In order to survive, the type 1 diabetic patient must have insulin. Many people with diabetes also need to control their blood pressure and cholesterol by taking medications.

In individuals with either type 1 or type 2 diabetes, diet and exercise are essential management. However, it is not easy to achieve and maintain the

desired glucose control. Therefore, oral agents are needed for attaining the goal of near-normal glycemia. At present, treatment for type 2 diabetes relies on several approaches mainly to reduce the hyperglycemia itself [14]. The oldest class of oral antihyperglycemic agents is sulfonylureas. As recently as 1994, sulfonylurea was the only available oral agent for the treatment of type 2 diabetes. It remains the most commonly used initial pharmacologic treatment for this disease because it is potent, effective, and inexpensive. Sulfonylureas work by binding to receptors on the surface of pancreatic islet beta-cells and stimulating the release of endogenous insulin [15]. However, sulfonylurea stimulation of insulin secretion is not glucose dependent, and hence hypoglycemia is an adverse effect of the treatment. Another problem with sulfonylurea is that after extensive treatment, many patients have shown secondary failure and no longer respond to sulfonylurea treatment [16]. There are several other therapies currently used as therapy for type 2 diabetes: biguanides, which reduce hepatic glucose production; thiazolidinediones, which enhance insulin action; and α-glucosidase inhibitors, which interfere with gut glucose adsorption.

Metformin is a biguanide, which was introduced in the United States in 1995 [17]. In contrast to sulfonylureas, metformin does not stimulate insulin secretion [18]. Adverse effects of metformin include gastrointestinal distress in up to 50% of patients [17]. The glucose lowering effect of metformin is generally equivalent when compared with sulfonylurea [19]. The α-glucosidase inhibitors were released in 1996. By inhibition of α-glucosidase, these inhibitors delay intestinal carbohydrate absorption and lower postprandial glucose excursions

[20]. The efficacy of α-glucosidase inhibitors is considerably less than that of either sulfonylurea or metformin [21]. Adverse effects of α-glucosidase inhibitors include flatulence, abdominal discomfort, and diarrhea. Currently, thiazolidinediones are represented by rosiglitazone and pioglitazone. The most prominent effect of thiazolidinediones is increased insulin-stimulated glucose uptake by skeletal muscle cells [22]. Adverse effects of thiazolidinediones include weight gain, edema, and anemia [23]. Mechanism of action and adverse effects of these oral agents is summarized in Table 1.1. As reviewed here, current available therapies have significant side effects and have limited efficacy [24]. Therefore, newer physiological approaches are desperately needed for treatment of type 2 diabetes. The use of hormones, other than insulin, is a relatively new area of clinical investigation and therapy for the treatment of diabetes.

1.2 Glucagon-Like Peptide-1

1.2.1 Incretin

The use of hormones, other than insulin, is a relatively new area of clinical investigation and therapy for the treatment of diabetes. It is possible to prevent the risk of hypoglycemia by potentiating insulin secretion in a glucose-dependent manner. In 1930, La barre introduced the term 'incretin' to describe the activity of gut that might enhance the endocrine secretion of pancreas [25]. In response to the ingestion of food, both insulin and insulin counter-regulatory hormones are released into the blood stream.

Insulin secretion is related to the serum concentration of absorbed nutrients, such as glucose, amino acids, and fatty acids. Insulin secretion is also

Table 1.1 Mechanism and adverse effects of the oral agents for type 2 diabetes.

Class	Mechanism of action / Adverse effect
Sulfonylureas	Increase in insulin secretion / Hypoglycemia
Biguanides	Decrease in hepatic glucose production Decrease in peripheral insulin resistance / GI disturbances, Lactic acidosis
Thiazolidinediones	Decrease in insulin resistance / Anemia, Hepatic toxicity
α-glucosidase inhibitors	Decrease in gut glucose adsorption / GI disturbances

influenced by incretins. The incretins are intestinal hormones that augment insulin secretion in the presence of elevated glucose levels [26]. In humans, two gastrointestinal peptide hormones are thought to be responsible for the incretin effect [27]. One is glucose-dependent insulinotropic polypeptide (GIP). GIP was originally named 'gastric inhibitory polypeptide' [28]. GIP was discovered in 1973 on the basis of its ability to inhibit acid secretion, but its insulinotropic properties were discovered soon after [29]. GIP is a peptide of 42 amino acids processed from a precursor of 153 amino acids [30]. As the cloning of cDNAs encoding the preproglucagons of the anglerfish was accomplished in the early 1980s, the second incretin hormone was discovered [31]. Shortly after cloning the proglucagon cDNAs of anglerfish, the proglucagon cDNAs of human and other mammals were cloned [32]. GLP-1 is synthesized in the intestinal L-cells by posttranslational processing of the glucagon precursor, preproglucagon, and is released into the circulation in response to a meal [33]. The origin of GLP-1 is depicted in Figure 1.1.

GIP is known to be secreted from specific endocrine cells, the so-called K cells, but recent studies have indicated that GIP cells are found in the entire small intestinal mucosa [34]. Secretion of GIP is stimulated by carbohydrates and by lipids. Therefore, GIP secretion is greatly increased in response to meal ingestion, resulting in a 10- to 20-fold increase in the plasma concentration [35]. Interaction of GIP with its receptor on the pancreatic beta cells causes an increase of cAMP levels, which in turn increases the intracellular calcium concentration and enhances the exocytosis of insulin granules [36]. The incretin

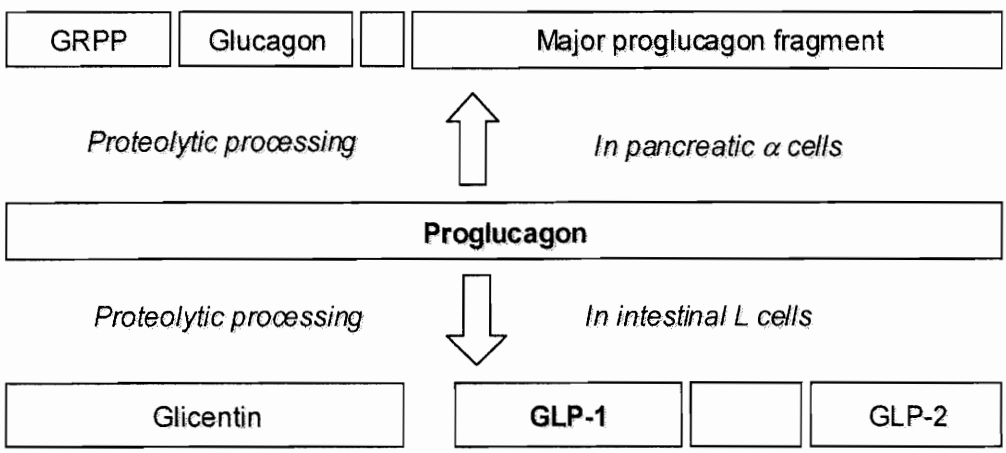

Figure 1.1 Origin of GLP-1. Proglucagon is cleaved into glucagons-like peptides and other peptides in intestinal L cells.

function of GIP was first suggested by one study [37] and was confirmed in detailed clamp-studies by another study [38]. Mice with a targeted deletion of the GIP receptor gene become glucose intolerant [39]. Immunoneutralization experiments showed that intestinal extracts contain potent insulinotropic agent in addition to GIP [40]. Furthermore, a study in patients with resections of different parts of the small intestine showed that the incretin effect does not correlate to the secretion of GIP, and that the distal small intestine releases an additional incretin hormone [41, 42]. It is well known that GLP-1 isoforms GLP-1(7-37) and GLP-1(7-36)amide are the bioactive insulinotropic peptides derived from preproglucagon [43]. GLP-1(7-37) and GLP-1(7-36)amide sequence as active forms are described in Figure 1.2.

Studies of the secretion of GLP-1 and GIP in type 2 diabetic patients have shown that meal-induced GIP secretion is normal or near-normal, whereas GLP-1 secretion is significantly impaired [43]. Potential therapeutic use of GLP-1 for type 2 diabetes was derived from the study demonstrating that the glucose-lowering actions of GLP-1 are preserved in patients with type 2 diabetes. To compare insulinotropic actions of exogenous incretin hormones (GIP and GLP-1), normal and type 2 diabetic patients participated. The effect of GIP in type-2 diabetic patients was significantly lower than in normal subjects [44], whereas GLP-1 retains the glucose-lowering effects in type 2 diabetic patients [45]. GLP-1 was capable of restoring insulin responses to glucose in type 2 diabetic patients.

GLP 1 (7-36) amide

His-Ala-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Val-Ser-Ser-Tyr- Leu-Glu-Gly-Gln-Ala-Ala-Lys-Glu-Phe-Ile-Ala-Trp-Leu- Val-Lys-Gly-Arg-NH_2

GLP 1 (7-37)

His-Ala-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Val-Ser-Ser-Tyr- Leu-Glu-Gly-Gln-Ala-Ala-Lys-Glu-Phe-Ile-Ala-Trp-Leu- Val-Lys-Gly-Arg-Gly

Figure 1.2 Active forms of GLP-1 are amide and glycine extended form.

1.2.2 Biological Effects of GLP-1

Binding of GLP-1 to the receptor causes activation of adenylate cyclase resulting in the formation of cAMP [46]. It has been known that all of the actions of GLP-1 are secondary to the formation of cAMP. Subsequent activation of protein kinase A leads to altered ion-channel activity, intracellular calcium^{2+} handling and enhanced exocytosis of insulin-containing granules [47]. A certain level of glucose must be present for GLP-1 to have any effect on insulin secretion. In addition, GLP-1 potentiates strongly the insulinotropic actions of glucose itself. Conversely, it seems that GLP-1 is required for glucose to exert its activity. Thus, in experiments in single beta cells, neither glucose nor GLP-1 alone affects intracellular calcium levels or membrane potential, whereas together they bring about a strong activation [48, 49].

GLP-1 conveys glucose competence to the beta cells [48]. The effects of glucose and GLP-1 may converge at the level of the adenosine tris phosphate (ATP) channels of the beta cells. These channels are sensitive to the intracellular K_{ATP} levels and thereby to glucose metabolism of the beta cells, but may also be affected by protein kinase A activated by GLP-1 [50, 51]. At any rate, it is of potential clinical importance that sulfonylureas, which bind to and close the KATP channels and thereby cause an influx of calcium, may uncouple the glucose dependency of GLP-1. Thus, GLP-1 administration to isolated perfused rat pancreases at low-glucose perfusate concentrations, which normally does not affect insulin secretion, resulted in dramatic stimulation of insulin secretion after pretreatment with sulfonylurea drugs [52]. It is has been shown that cAMP

generated by activation of the GLP-1 receptor may also influence the exocytotic process directly and this process has been estimated to account for up to 70% of the entire secretory response. ATP may also influence the exocytotic process, which may therefore represent another site of convergence for the glucose and GLP-1-mediated signals [51].

Apart from its insulinotropic actions, GLP-1 has a number of effects, which makes it extremely desirable as type 2 diabetes treatment. Firstly, GLP-1 enhances insulin secretion only when glucose levels are high (>250 mg/dl) [53].

GLP-1 potentiates glucose-induced insulin secretion, but it does not have any effect on unstimulated insulin secretion. Therefore, it is unlikely to cause hypoglycemia. Secondly, it stimulates not only insulin gene transcription, but also all steps of insulin biosynthesis [54]. Thus, it helps to provide a continuous supply of insulin for secretion.

Also, it upregulates genes for the cellular machinery involved in insulin secretion [55]. In addition, GLP-1 has been shown to be capable of providing new β-cells in the subjects with insufficient cells [56], and proliferation of β-cells [57]. Recently, it has been found that GLP-1 receptor signaling results in a reduction of β-cell apoptosis [58], which will further contribute to increased β-cell mass [59]. Thirdly, GLP-1 strongly inhibits glucagon secretion. Inhibition of glucagon secretion is also glucose dependent [60]. As a result of the studies of GLP-1 infusion in diabetic patients without any residual β-cell secretory capacity, GLP-1 retains glucose-lowering activity due to strong inhibition of glucagon secretion [61]. Further effects of GLP-1 include inhibition of gastrointestinal

motility, especially gastric emptying [62]. The slower rate of gastric emptying is desirable in diabetic patients, because it reduces postprandial increase of glucose level. Taken together all these effects render GLP-1 very promising as a therapeutic agent for type 2 diabetes. The schematic of GLP-1 actions is in Figure 1.3.

1.2.3 Limitation of the Effects of GLP-1

However, there is a problem limiting the usefulness of GLP-1 in treatment. The major drawback for the use of GLP-1 as therapeutic agent is the extremely short half-life due to rapid degradation. The rapid initial degradation is due to the ubiquitously expressed enzyme, dipeptidyl peptidase IV (DPP-IV) [63]. It cleaves off the two N-terminal amino acid residues.

The conversion of intact, biologically active GLP-1 to its metabolites occurs with an apparent half-life of 1-2 minutes [64]. It has been shown that more than half of GLP-1 released postprandially circulates as the truncated metabolite. After subcutaneous injection of GLP-1, concentrations peak after 30-60 minutes and little remains in the circulation after 1-2 hours [65]. This means that the majority of exogenous GLP-1 is present in the circulation as the truncated metabolite.

1.2.4 Strategies for Clinical Applications of GLP-1

It has been reported that intravenous infusions of GLP-1 completely normalize blood glucose in type 2 diabetic patients [66]. Because continuous

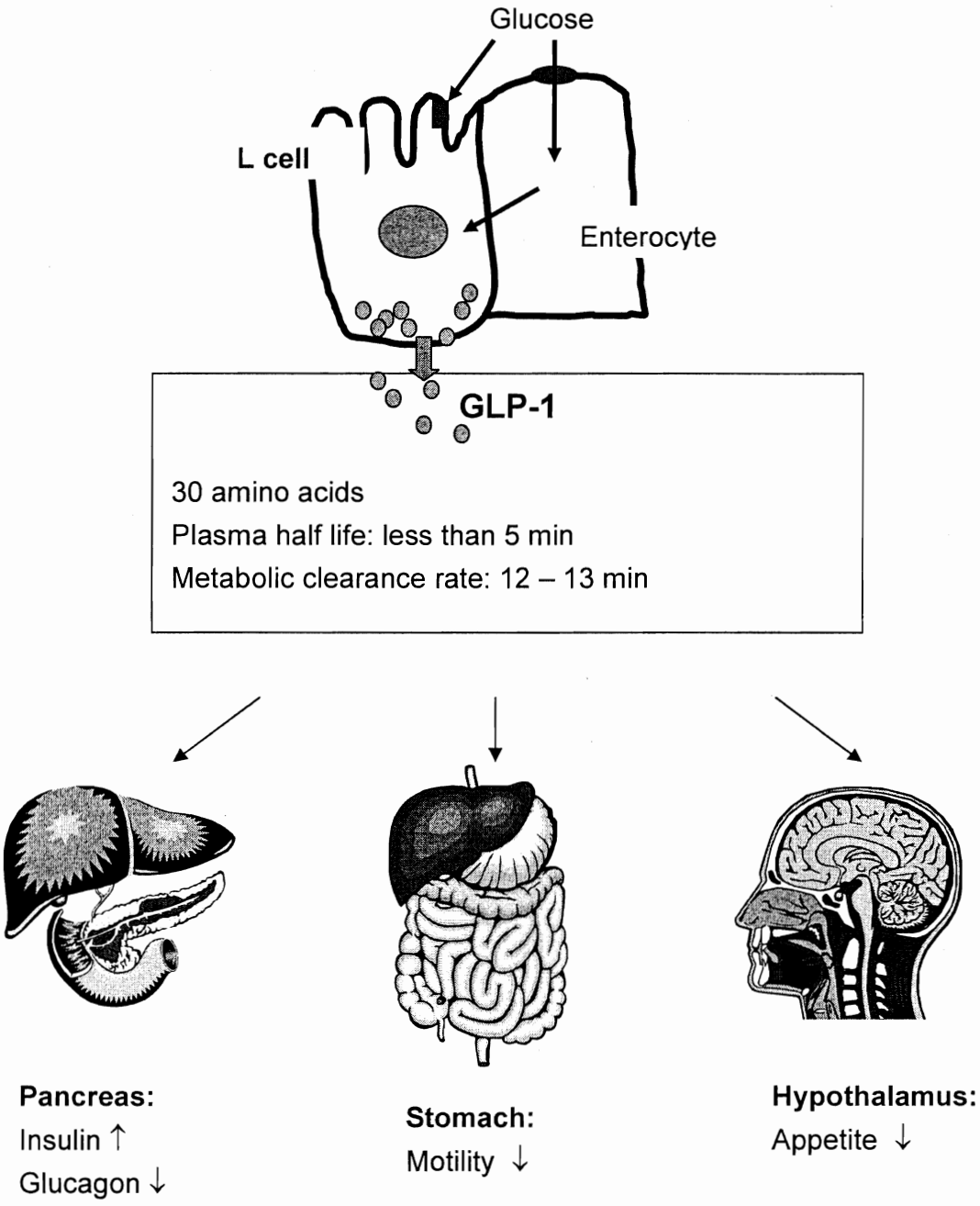

Figure 1.3 Actions of GLP-1 in pancreas, stomach and hypothalamus.

intravenous infusion is not suitable for routine administration, many researchers have tried various other types of administration and those studies have shown the effectiveness of GLP-1.

In order to delay the degradation of GLP-1, the properties of the injectable form have been modified. Possibilities include the preparation of GLP-1 with protamine or zinc, as has been done with insulin [67]. However, the results from using various methods have shown that the period before degradation is still too short to get therapeutic effects from GLP-1 in diabetic subjects. Therefore, multiple injections are required for therapeutic use of GLP-1. Although many practical problems still remain to be solved, a therapy based on GLP-1 is one of the most promising recent approaches to the treatment of Type 2 diabetes.

A number of new approaches are currently under investigation for using GLP-1 as therapeutic agent. Since the extremely short half-life is due to DPP-IV activity, DPP-IV resistant analogs have been developed as alternatives [68]. The enzymatic cleavage occurs at the alanine in position two; thus analogs modified at this position were more resistant than GLP-1 itself. Such analogs retain a prolonged insulinotropic activity compared with native GLP-1. An interesting analog is exendin-4, a peptide of 39 amino acids isolated from the venom of lizards, which has 53% sequence homology to GLP-1. Exendin-4 is a full agonist at the GLP-1 receptor [69] and is resistant to actions of DPP-IV. One study showed that once a day intraperitoneal administration for 13 weeks in diabetic mice almost normalized blood glucose and reduced hemoglobin to near normal values [70]. In another study, both blood glucose and hemoglobin levels were

improved and body weight was lowered following up to 42 days of exendin-4 treatment in experimental animal models of type 2 diabetes [71]. Also, it was demonstrated that 8 weeks exendin-4 treatment of Zucker fatty rats substantially improved glycemia, hyperinsulinemia and body weight [72]. Amylin pharmaceuticals presented data obtained in patients with type 2 diabetes treated with exendin-4, two or three times daily injection for 4 weeks, in addition to the conventional treatment [73].

More recently, numerous studies of exendin-4 treatment of diabetic patients have been published. After once or twice daily administration of exendin-4, the glucose level was significantly reduced in type 2 diabetic patients [74]. Also with combination of sulfonylurea and metformin, the postprandial glucose was significantly reduced in patients with type 2 diabetes [75]. One study showed that a single subcutaneous injection of exendin-4 has similar effects to overnight administration of intravenous GLP-1 [76]. In addition, exendin-4 given to diabetic patients before a mixed-nutrient liquid meal significantly lowered the postprandial glucose excursion [76]. In that study, exendin-4 appeared to be equally effective in lowering blood glucose in subjects with poorly controlled diabetes as it was in subjects with good glycemic control.

Another analog, NN2211 has been developed by NovoNordisk. An acyl chain was linked to the native GLP-peptide, so that the peptide binds to albumin. This analog can lower the sensitivity to DPP-IV and also delay its absorption from the injection site [77]. Acute or chronic administration of NN2211 reduced glucose and increased insulin levels, suppressed glucagon release, and delayed

gastric emptying in a glucose-intolerant animal model [78]. In patients with type 2 diabetes, a single subcutaneous injection of NN2211 reduced glucose levels to near-normal levels overnight and lowered postprandial glucose excursions by enhancing increased insulin secretion [79]. After 1 week treatment with NN2211, glycemia was markedly improved and endogenous glucose release was significantly reduced in patients with type 2 diabetes [79].

DPP-IV-resistant analogs exendin-4 and NN2211 have been shown to have the positive effects of GLP-1 in patients with type 2 diabetes. Also, their duration of action was extended to enable once or twice a day administration. Even though the use of DPP-IV-resistant analogs of GLP-1 shows considerable promise, it still requires frequent injection to achieve the therapeutic effect.

The second major strategy is to inactivate DPP-IV. The use of DPP-IV inhibitors was suggested as a result of extreme degradation of GLP-1 in type 2 diabetic patients [80]. Subsequently, numerous studies have indicated that administration of DPP-IV inhibitors can be offered to subjects at risk of developing diabetes. Several orally effective inhibitors of DPP-IV have been shown to have antidiabetic actions in various hyperglycemic animal models. For example, acute administration of DPP-IV inhibitors to Zucker fatty rats increased the percentage of intact GLP-1 from 20 to 100% [81]. Another study showed that after a 3-month treatment with a DPP-IV inhibitor, there was improved glucose tolerance as well as enhanced insulin secretion and action [82]. DPP-IV inhibition was also effective in obese, glucose-intolerant monkeys [83]. Several DPP-IV inhibitors are in clinical trials. Recently NVP-DPP728 was administered for 4

weeks in patients with type 2 diabetes with relatively mild hyperglycemia who were not treated with medications [84]. Fasting plasma glucose level was significantly reduced after treatment, and this treatment was well-tolerated. The new DPP-IV inhibitor LAF237 is similar to the previous compound. Single-dose efficacy studies in type 2 diabetic patients showed reduced glucose and glucagon level, and increased GLP-1 level [85]. After treatment for 4 weeks, metabolic control was significantly improved. LAF237 has reached phase 3 clinical trial [85].

Treatment with the DPP-IV inhibitor significantly reduced glucose levels throughout the day, and insulin levels were also reduced. It is advantageous to use DPP-IV inhibitors because there are no adverse effects, and it is orally available. However, it is unclear whether DPP-IV inhibition will have long-term effects. Because DPP-IV metabolizes a large number of regulatory peptides, the effect of inhibitors cannot be entirely attributed to GLP-1. For example, since GLP-2, an intestinal growth factor, is also a substrate of DPP-IV, DPP-IV inhibition may have an effect on intestinal proliferation [80].

1.3 Polymeric Drug Delivery Systems

1.3.1 Overview of Polymeric Drug Delivery Systems

Polymeric drug delivery is the most widely studied area of drug delivery in recent years. Polymeric systems in a matrix or reservoir form can offer sustained and controlled release of drugs. Another advantage is that polymers can be manipulated to possess certain properties for physiological criteria. Advantage of

polymeric drug delivery systems may provide advantages such as increased efficacy and reduced side effects and toxicity [86].

Drug therapy via the intravenous route of administration is the most direct way to achieve target concentrations in serum. However, this method has several disadvantages including dose limitations, toxicity, and low availability. Therefore, multiple injections are required for effective treatment.

Recently, many therapeutic proteins, peptides, or DNA-based drugs have become available due to advances in biotechnology. These therapeutic macromolecules have drawn increasing attention in drug delivery. Conventional routes of administration of these drugs require frequent injection to achieve therapeutic concentration in blood, because they have very short half-lives in blood plasma and are susceptible to physical or chemical degradation. This results in poor patient compliance and possible side effects [87-89]. As a result, biomedical polymers are designed to solve these problems. In order to reduce the frequency of injection of peptide and protein drugs, numerous attempts have been made to develop implantable or injectable systems for sustained and controlled release. Polymeric systems may also protect against the harsh environment when delivering peptides and proteins orally.

Also, polymeric drug delivery systems can be applied to targetable delivery systems including nonviral gene delivery carriers. Biodegradable polymers and stimuli-sensitive polymers are the keys to designing suitable delivery systems for bioactive agents. In the subsequent sections, aspects of

biodegradable polymers, thermosensitive polymers, and non-viral gene carriers will be discussed.

1.3.2 Biodegradable Polymers

Many synthetic and natural polymers have been studied for implantable drug delivery applications. Implantable polymers can be inserted into the body by a surgical procedure. Biocompatibility is required for the implantable polymer. Biodegradable polymers are better for implants because there is no need for surgical removal. Poly (lactic acid), poly (glycolic acid), poly (caprolactone), polyanhydrides, poly (phosphazines) and poly (orthoesters) are examples of implantable polymers.

For injectable delivery systems, poly (lactide-co-glycolide) (PLGA) was tested in the water miscible solvent glycofurol. When it is injected subcutaneously, the solvent diffuses into the bloodstream and its serum level per day fits FDA guidelines [87]. Also, poly (ortho-ester) semisolid formulation can be used as an injectable system [88]. Because it can be used under mild conditions, it is suitable for protein delivery. Biodegradable microspheres are injectable and suitable for protein/peptide delivery [89, 90]. Lupron Depot® (leuprorelin acetate/PLGA; Takeda/Abbott) for male prostate cancer [91] and Nutropin Depot® (human growth hormone/PLGA; Genentech/ Alkermes) [92] were both approved for human use. Most of all, gel-forming biodegradable and thermosensitive polymers are suited for peptide and protein delivery due to many favorable characteristics [93-95]. This will be discussed in the next sections.

1.3.3 Thermosensitive Polymers

1.3.3.1 Nonbiodegradable Thermosensitive Polymers

For numerous biomedical applications and drug delivery systems, various approaches have been made to design polymers which respond dramatically around a certain transition temperature in an aqueous environment [96-113]. Some polymers exhibit lower critical solution temperature (LCST) in water, above which the polymer is not soluble in water. Among those polymers, poly (N-isopropylacrylamide) (NIPAAm) and its copolymers have been extensively studied because their LCSTs are between body and room temperature. Below the LCST, the enthalpy term contributed by the hydrogen bonding between water and polar groups of the polymer causes the polymer to stay dissolved. Above the LCST, the hydrophobic interaction, which is an entropic term, becomes a major factor that triggers the polymers to collapse. Also a shift in the C-H stretching band was observed on the nanoscale level via atomic force microscopy [96].

Interestingly, the LCST of poly (NIPAAm) can be controlled via copolymerization with monomers having different degrees of hydrophobicity. It is known that higher LCST can be obtained by incorporating a hydrophilic monomer, while copolymerizing with a hydrophobic monomer results in lower LCST [96].

Another kind of thermosensitive polymer is poloxamer, poly (ethylene oxide)-poly (propylene oxide)-poly (ethylene oxide) (PEO-PPO-PEO), which is commercially available [114]. Poloxamers are a series of surfactant polymers. Poloxamer 407 (Pluronic F127) can form micelles at low concentrations in water. As the concentration increases, it can form a hydrogel at body temperature via

packing of the micelles. Therefore, an aqueous solution of this polymer may be used as a parenteral drug delivery depot. However, disintegration of this depot happens very quickly (within 1 day) after dilution. Hence, it cannot be used for sustained release [114].

Overall these nonbiodegradable polymers are not suitable for parenteral applications due to lack of biocompatibility.

1.3.3.2 Biodegradable Thermosensitive Polymers

A biodegradable and thermosensitive polymer, low molecular weight triblock copolymer of PLGA and poly (ethylene glycol) (PEG) was designed. It is a hydrophilic/hydrophobic balanced polymer [93]. After subcutaneous injection of this polymer in rats, the temperature-induced sol-gel transition of aqueous solution was observed [94]. After an intratumoral injection of an injectable formulation of PLGA-PEG-PLGA (ReGel^{TM}) and paclitaxel, Oncogel^{TM} (Macromed, Inc.), the water-insoluble drug was solubilized and slowly cleared from the injection site with minimal distribution into other organs [95]. This system is currently undergoing Phase II human clinical trials. This system is particularly suitable for sustained release of unstable peptide or protein drugs due to the absence of harsh processing conditions. It has been shown that sustained release of insulin can be achieved for 2 weeks by a single subcutaneous injection [115, 116].

1.3.4 Peptide and Protein Release Systems

Peptide and protein drugs have short *in vivo* half-lives, physical and chemical instability, and the low oral bioavailability. Thus, frequent injections as solution have been required for peptide and protein drug delivery. One way to solve this problem is using an injectable biodegradable polymer depot. Injectable biodegradable copolymers of lactic and glycolic acids (PLGAs) as controlled-release systems for the delivery of peptide and protein drugs and vaccine antigens have been studied extensively [87-89].

1.3.4.1 Biodegradable Microspheres

As explained earlier, frequent injections have been required for peptide and protein drugs because of their physical and chemical characteristics. It has been more than 20 years since the report of a sustained release of a microencapsulated protein. However, it cannot be used clinically because of the instability of the protein drug. In recent years, several therapeutic proteins have been developed due to advances in stabilization. For example, human growth hormone was formulated for clinical testing for monthly administration [88]. Still, there are problems to overcome for using protein-loaded microspheres, which include a "burst effect" and incomplete protein release [89].

1.3.4.2 Injectable Depot Systems Via Sol-gel Transition

Most of injectable microspheres or implant systems require complicated fabrication processes using organic solvents for biodegradable polymers for

peptide and protein drug delivery. The use of organic solvents can cause denaturation when protein drugs are to be encapsulated [89]. In addition, the solid form requires surgical insertion, which can cause tissue damage or irritation. These problems can be solved by using a thermosensitive, biodegradable hydrogel consisting of blocks of poly (ethylene oxide) and poly (L-lactic acid). These copolymers exhibited temperature-dependent reversible gel-sol transitions. Bioactive therapeutics can be loaded into this hydrogel, which is aqueous at elevated temperature. The polymer can be injected when it is in the sol state. After subcutaneous injection, the temperature of injected polymer lowers to body temperature. Then, the copolymer with therapeutics forms a gel that can act as a sustained-release matrix for drugs [93].

Careful manipulation of block-lengths of PEG and PLGA led to the discovery of lower transition in the triblock copolymers of PEG and PLGA. The hydrophilic and hydrophobic balanced triblock copolymer, PEG-PLGA-PEG, exhibited sol-to-gel transition and gel-to-sol transition when temperature was increased [117]. Importantly, the lower transition can be used for drug delivery applications because the polymer is a sol at room temperature and becomes a gel at body temperature.

Another type of triblock copolymer, PLGA-PEG-PLGA is also a biodegradable, biocompatible polymer that showed reverse thermal gelation properties [95, 118]. The polymer (ReGel™, Macromed, Inc.) shows a unique characteristic which is a sol at room temperature and a gel at body temperature. This polymer forms a hydrogel above the transition temperature, and this

hydrogel interacts physically via hydrophobic domains. Dynamic mechanical analysis revealed that an increase in viscosity of approximately four orders of magnitude accompanies the sol-gel transition [95]. This ABA-type triblock copolymer was used as a drug delivery carrier for continuous release of human insulin [115, 116]. The release of human insulin from ReGel™ exhibited no initial burst and a constant release rate *in vitro* due to the domain structure of the gel and modification of the association states of insulin by zinc. Animal studies using both Sprague-Dawley rats and type 2 diabetic Zucker diabetic fatty rats were performed to determine the *in vivo* release profile of insulin from ABA block copolymer. In both studies, ReGel™ formulation maintained insulin secretion for 2 weeks. Therefore, only twice a month injection would be required for diabetic patients to maintain basal insulin levels [115]. Further discussion on gelation and drug release mechanisms will be provided in Chapter 2.

1.3.5 Targetable Polymers

For design of a targetable polymeric carrier, a water soluble polymer can be used with targeting moieties. Poly [N-(2-hydroxypropyl) methacrylamide] (HPMA) is a representative example. Doxorubicin has been conjugated to HPMA and studied for cancer therapy. The drug can be linked via a peptide spacer specifically recognizable by lysosomal enzymes due to the chemical modification of the side chain. Upon endocytosis, drugs are localized and released within the cells. By using this polymer, side effects were reduced, and efficacy was improved with a dramatic decrease in multidrug resistance [119].

1.3.6 Nonviral Carriers for Plasmid DNA

Recently, non viral polymeric gene carrier systems have been extensively studied. Positively charged polycationic polymer can form complexes with the negatively charged plasmid DNA via electrostatic interactions. A variety of targeting moieties may be attached to the polymer. For example, conjugation of lactose through a poly (ethyl glycol) (PEG) spacer to poly (L-lysine) (PLL) showed enhanced transfection in cultured HepG2 cells [120].

As a nontoxic carrier, poly [alpha-(4-aminobutyl)-L-glycolic acid] (PAGA) has been developed. PAGA is a polycationic polymer with hydrolytically degradable linkages [121]. It was used as a delivery carrier for IL-10 plasmid, for prevention of autoimmune insulitis in NOD mice [122]. Also, after repeated injections of PAGA/pmIL-12 complexes, the survival rate was significantly improved and tumor growth was inhibited in BALB/c mice bearing subcutaneous tumors [123]. Further reviews on nonviral gene delivery are placed in the following section 1.4.

1.4 Nonviral Gene Delivery

1.4.1 Gene Delivery

It has been more than three decades since the concept of gene therapy was originally developed [124]. The major aim of gene delivery is restoration, modification or enhancement of cellular function by introducing genetic material into a target cell. The genetic material can be either DNA or RNA. The altered cellular function can result in increase or decrease of protein production. More

than 600 clinical trials have been started with more than 3500 patients worldwide. Several products are in Phase II and Phase III stages [125, 126], and there already exists one commercial antisense product, Vitravene, a phosphorothioate oligodeoxynucleotide for the treatment of an inflammatory viral infection of the eye caused by cytomegalovirus [127]. In spite of promising preclinical data, the early clinical trials failed to demonstrate efficacy, mainly because of a lack of vectors capable of efficiently introducing the therapeutic gene into the target cells of humans.

DNA delivery approaches can be classified into viral and non-viral. Over the past decade, numerous approaches have been proposed and developed for transferring genes to various tissues with viral and nonviral carriers, but all have limitations. Currently, viral systems are more effective because they achieve higher efficiencies for delivery and expression. However, viruses present serious problems: they are expensive to produce, present quality control problems, potentially damaging to the cells they infect, and may cause potent immune responses that limit their use [128]. In addition, there is always a possibility of replication. For these reasons, it may be desirable to develop non-viral systems. Nonviral gene delivery vectors offer numerous advantages over viral vectors: they have excellent safety profiles, they can carry large amounts of DNA, and are less problematic in terms of quality control [129].

1.4.2 Nonviral Vectors for Gene Delivery

1.4.2.1 Cationic Polymers

Cationic polymers have been used since the late 1980s [130] for gene delivery. Recently, nonviral gene delivery systems based on complexes of condensed DNA with cationic polymers have attracted great attention. The complexes of condensed DNA with cationic polymers not only can protect DNA from nuclease degradation but also have a small enough size to enter the cell through endocytosis. Cationic polymers contain high densities of primary amines, which are protonatable at neutral pH. The high density of positive charges allows the cationic polymers to form stable complexes with DNA. The cationic polymers self-assemble with DNA to generate condensed structures capable of entering the cell.

Polyethylenimine (PEI) is a cationic polymer that has proven to be an effective transfection agent [131-133], and it has been widely used in non viral gene delivery. It has a high cationic charge density resulting from the protonatable amines on every third carbon [134]. Due to the high cationic charge density, it is able to efficiently condense DNA and has a high intrinsic endosomal activity as compared to other polycations [133]. PEI can be synthesized by the acid-catalyzed ring opening polymerization of aziridine as either a linear or a branched structure [135]. It has been shown that low molecular weight PEI is less cytotoxic than high molecular weight PEI [136]. PEI/DNA complexes must also bear a net positive charge in order to efficiently transfect cells [134, 136, 137]. PEI/DNA complexes form spheres or toroids with mean diameters ranging from

30 to 100 nm. *In vivo* gene transfer with PEI was reviewed by Remy et al. [138]. The highest transfection efficiency was observed when net charges are around neutrality after intracranial injection in mice [134]. Boletta et al. studied gene delivery to rat kidneys with three different forms of PEI [139]. Transfection efficiency was highest with moderately positively charged complexes. Branched PEI has been modified with polyethylene glycol (PEG) [140], targeting groups [120, 141], and nuclear localization sequences [142, 143]. Kissel et al. grafted PEG to branched PEI (molecular weight 25,000) [140]. The degree of PEGylation and molecular weight of PEG affected DNA condensation and bioactivity of polymer/DNA complexes.

Water soluble lipopolymer (WSLP) is branched PEI (molecular weight 1800) conjugated to cholesterol on a primary amine. Typically 0.8-1.2 cholesterol molecules are conjugated per one PEI. Cholesterol increases the degree of transgene expression greatly [144]. Lipopolymers condense plasmid DNA by hydrophobic interactions as well as electrostatic interactions. In addition, the lipid coating on plasmid DNA increases its permeability through cell membranes [144]. WSLP has been studied and shown effects for the treatment of diseases including ischemic myocardium [145], diabetes [146], and cancer [147, 148].

Poly-L-lysine (PLL) has been used for condensation of DNA under various salt conditions. However, PLL/DNA complexes are very unstable under physiological conditions. Modifications of PLL to enhance efficiency of gene delivery have been made in various ways. To minimize aggregation, lactose-poly(ethylene-glycol)-grafted poly-L-lysine (Lac-PEG-PLL) polymers were

synthesized [149]. Due to grafted PEG, Lac-PEG-PLL has higher solubility. For gene delivery into rat hepatocytes, galactosylated poly(L-lysine) (Gal-PLL) was synthesized [150]. By using different molecular weights of PLL and conjugation ratio of galactose, six different types of Gal-PLL were synthesized. Similarly, mannosylated poly(L-lysine) (Man-PLL) was synthesized [151]. Man-PLL was shown to express genes in murine macrophages from peritoneal exudates and macrophages in the liver and spleen. Based on stearyl-poly(L-lysine) (stearyl-PLL) and low density lipoprotein (LDL), PLL was modified as the terplex system. This supramolecular gene carrier is intended to balance the hydrophobic and electrostatic interactions between stearyl-PLL, LDL, and DNA [152]. PLL-arabinogalactan was also examined for gene delivery [153].

Another type of structure that has been examined for gene delivery is the dendrimer. Dendrimers can be either intact or fractured. Intact dendrimers have two arms from every branch point, whereas fractured dendrimers have zero, one, or to arms from each branch point [149]. Dendrimers can condense plasmids by electrostatic interactions of their terminal primary amines with the DNA phosphate groups. Gene transfer efficiency of these complexes is influenced by dendrimer concentration in the complexes. Polyamidoamine (PAMAM) dendrimers are highly branched spherical polymers whose surface charge and diameter are determined by the number of synthetic steps [154]. The structure of dendrimers is related to the core molecules. The stepwise polymerization begins with core molecules, either ammonia or ethylenediamine.

Chitosan is a cationic monosaccharide that is biocompatible and

resorbable. Chitosan based gene delivery has shown low toxicity and little liver accumulation when low molecular weights are used [155]. It has also been shown that chitosan/DNA complexes can transfect various cell types with varying results [156, 157].

Biodegradable polymers such as poly[α-(4-aminobutyl)-L-glycolic acid] (PAGA) [158, 159], poly(D,L-lactic acid-*co*-glycolic acid) (PLGA) [160, 161], PEG-PLGA-PEG [162], and PEG-PLL-*g*-His multiblock copolymers [163] have been used as non viral gene delivery carrier. PAGA, the biodegradable and water-soluble polymer, condenses DNA and subsequently releases DNA upon hydrolysis of the polymer [160]. The complex showed higher *in vitro* gene transfection efficiency with lower cytotoxicity than poly-L-lysine. PAGA has been used for interleukin-4 and interleukin-10 delivery for the treatment of diabetes [122, 164]. Plasmid DNA with PEG-PLGA-PEG has shown zero-order release of the plasmid up to 12 days [162]. A biodegradable multiblock-copolymer PEG-PLL-*g*-His was synthesized and used for plasmid delivery [163]. The number of cells transfected with this polymer showed a 5-fold increase above the PLL control [163].

1.4.2.2 Cationic lipid

Cationic lipid and neutral phospholipids such as DOPE [165] are used for gene delivery also. DNA complexes with cationic lipid enter cells by direct fusion with plasma membrane, and further fuse with endosomal or lysosomal membrane. After nuclear localization, plasmid DNA is maintained in an

extrachromosomal form [166]. The self-assembly process of these complexes requires interaction between lipid molecules and also interaction with the DNA [167]. The size and charge of the lipid/DNA complex can be optimized by changing the composition of complex; thus the efficiency of gene delivery can be optimized as well. However, the ability of control in lipid dispersion is generally limited [168]. The complexes of lipid and DNA are often poorly water soluble and their macroscopic characteristics are unstable over time, limiting their applications [169]. Cholesterol has been used for gene delivery in the form of 3β [*N*-(*N',N'*-dimethlaminoethane)-carbamoyl] cholesterol (DC-Chol). Liposomes made from DC-Chol/DOPE have been used for neuron cell transfection *in vitro* [170].

1.4.3 Barriers to Effective Nonviral Gene Delivery

The current nonviral gene delivery systems have shown low transfection efficiency [129]. Transfection efficiency is dependent on the efficiency of DNA delivery and the efficiency of DNA expression. Currently much effort is devoted to optimizing transfection efficiency of nonviral vectors. In the past decade, numerous papers have described the barriers to effective DNA delivery [171-174]. These barriers include (1) physical and biochemical degradation in the extracellular space, (2) the extracellular matrix and cell surface interactions, (3) internalization, (4) trafficking from endosomes to lysosomes and subsequent degradation, (5) escape from endosomes into the cytoplasm, (6) dissociation of DNA from its carrier, (7) transfer into the nucleus, and (8) transcription for gene

transfer (or hybridization for antisense inhibition). The gene delivery pathway is depicted in Figure 1.4.

There are three major barriers to DNA delivery into the cell [175]. The first major barrier is low uptake across the cell membrane. Cell membranes are negatively charged because of anionic sialic acid groups on the surface [176]. Negatively charged DNA molecules are condensed with cationic transfection reagents and form complexes. The uptake of polymer/DNA complexes can be achieved by the electrostatic interactions between the complexes and sialic acid groups. In addition, these complexes are taken up by cells through endocytosis, pinocytosis, or phagocytosis [177]. For effective DNA delivery, endocytosis chould be increased by a receptor-mediated targeting. The transferrin receptor was first investigated for receptor-mediated gene delivery [178]. All actively metabolizing cells require iron that is internalized by the cell as a transferrin-iron complex by means of receptor-mediated endocytosis. Another well studied ligand for potential use in receptor-mediated gene delivery is asialoglycoprotein [179]. This was the first ligand to be conjugated to polylysine and used to specifically target cells through a receptor.

The second major barrier is inadequate release of DNA with limited stability. Due to the low pH and presence of enzymes within endosomes and lysosomes, DNA can be degraded easily. One of the most common methods for increasing the *in vitro* transfection efficiency of polymer/DNA complexes is to treat the cells with chloroquine [180]. Chloroquine is thought to have a buffering capacity that prevents endosomal acidification, leading to swelling and bursting

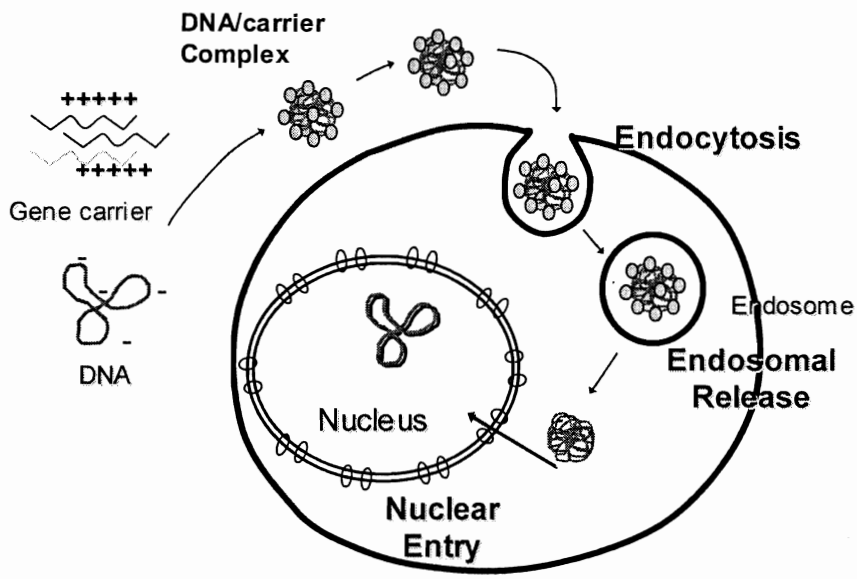

Figure 1.4 Illustration of gene delivery pathway from outside of cell into nucleus.

of the endosomes and has been shown to enhance the transfection activity of polycation/DNA complexes [180]. Because of toxicity, this approach is limited to *in vitro* applications. Adenovirus particles have been used to enhance transfection of polymer/DNA complexes, but it is unlikely to be widely used *in vivo* because it may provoke inflammatory responses [181, 182]. Instead of using whole virus particles to enhance cytoplasmic delivery, fusogenic peptides have been used [183]. PEI has been known to possess intrinsic endosomalytic ability [131]. It has been suggested that this protonation in the endosome leads to endosomal swelling and bursting with subsequent enhancement of transfection activity.

The third major barrier is lack of nuclear targeting. It has been known as the most limiting barrier to non viral gene delivery. An early report showed 50–100% of mouse embryonic fibroblast thymidine kinase-negative ($LMTK^-$) cells expressed kinase activity when the *Herpes simplex* virus thymidine kinase (HSV-TK) gene was injected directly into the nucleus, compared with <0.01% when plasmid was injected into the cytoplasm [184]. Translocation of macromolecules across the nuclear envelope occurs via pores that serve as size-exclusion barriers. Small molecules enter the nucleus by passive diffusion while larger macromolecules only enter via highly regulated active processes. Small oligonucleotides (ODNs), 18–28 bp in size, have been shown to rapidly and preferentially accumulate into the nucleus after transfection [185, 186] and the size limit for passive diffusion of ODNs was found to be between 200 and 310 bp [187]. Transport of macromolecules should proceed through specialized nuclear

envelope spanning channels called nuclear pore complexes (NPCs) [188, 189]. Selective nuclear import of macromolecules larger than 40 kDa is mediated by nuclear localizing signals (NLSs). The nuclear localizing signal is recognized by a heterodimeric protein complex of importin-α and importin-β. Importin-α then interacts directly with the cargo NLS, whereas importin-β docks the complex to the NPC by specifically binding to a subset of hydrophobic phenylalanine–glycine-rich repeats [190, 191]. Therefore, larger nucleic acids and plasmids without nuclear targeting component cannot enter the pores passively.

1.5 Rationale for Study

Glucagon-like peptide-1 (GLP-1) is an insulinotropic hormone produced by intestinal L-cells. It has been proposed that GLP-1 can be used as a new treatment for type 2 diabetes mellitus because it stimulates insulin secretion and its effectiveness is maintained in type 2 diabetic patients. In spite of its many remarkable advantages as a therapeutic agent for diabetes, GLP-1 is not immediately clinically applicable because of its extremely short plasma half-life. Numerous approaches have been attempted to solve this problem, but these results have shown that the period before degradation is still too short to get therapeutic effects from GLP-1 in diabetic subjects. Therefore, multiple injections are required for therapeutic use of GLP-1. Although many practical problems still remain to be solved, a therapy based on the GLP-1 is one of the most promising recent approaches to the treatment of type 2 diabetes.

The main aim of this study was to design a new and effective GLP-1 delivery system for therapeutic use for type 2 diabetes treatment. The first part of this study was based upon sustained release of GLP-1 using a biodegradable injectable polymer. Proteins or peptides delivery using polymers are currently available for therapeutic applications. One of the popular methods for protein or peptide delivery is to entrap them in biodegradable polymers to achieve sustained and controlled release rates. However, as a result of employing harsh preparation conditions as well as the organic solvent, loaded proteins or peptides can undergo conformational changes that lead to decreased structural integrity and compromised biological activity. One way to overcome these problems is to use polymer hydrogels formed by sol-gel transition in water. However, nonbiodegradable polymers exhibit moderate toxicity *in vivo*. In this study, a thermosensitive and biodegradable low-molecular weight PLGA-PEG-PLGA that exhibits sol-gel transition property in water was used as a depot for the delivery of GLP-1.

In Chapter 3, construction of effective GLP-1 plasmid and its delivery were also studied for therapeutic use of GLP-1 for type 2 diabetes treatment. As mentioned earlier, nonviral gene delivery is more desirable than viral gene delivery system because nonviral gene delivery vectors have excellent safety profiles, and can carry large amounts of DNA. However, the efficiency of nonviral gene delivery system is not sufficient for therapeutic applications. Since the expression levels of the introduced gene depend mainly on the strength of transcriptional regulatory elements, GLP-1 plasmid was constructed by using

optimal transcriptional regulatory elements. Also, GLP-1 plasmid was further modified to enhance nuclear import, because the most limiting barrier to non-viral gene delivery is lack of nuclear targeting.

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CHAPTER 2

CONTROL OF BLOOD GLUCOSE BY NOVEL GLP-1 DELIVERY SYSTEM USING PLGA-PEG-PLGA IN ZUCKER DIABETIC FATTY RATS

2.1 Introduction

Polymeric drug delivery has been very extensively studied in recent years [1]. Due to advances in biotechnology, many therapeutic protein and peptide drugs are available. Peptide and protein drugs have shown limited bioavailability due to their physico-chemical limitations. Thus, parenteral administration is the common delivery route of these therapeutics. The conventional route of administration such as intravenous injection, intravenous infusion, and subcutaneous injection require frequent injections to achieve therapeutic concentrations in blood. Frequent administration causes patient discomfort and inconvenience. To improve the quality of therapy, frequency of injection should be reduced. If drug is released in a sustained manner, frequency of dosing can be reduced. In addition to improving patient comfort, less frequent injections will smooth out the plasma concentration-time profiles. It will help to reduce adverse events and side effects. Significant effort has been made to develop implantable

or injectable parenteral devices for sustained and controlled release of protein drugs in order to reduce the frequency of injection [1]. For example, glucagons-like peptide-1 has a very short *in vivo* half-life due to rapid degradation by the enzyme dipetidyl peptidase-IV (DPP-IV). Continuous administration is required because of the short half-life. Alternative approaches are necessary for the clinical application of GLP-1.

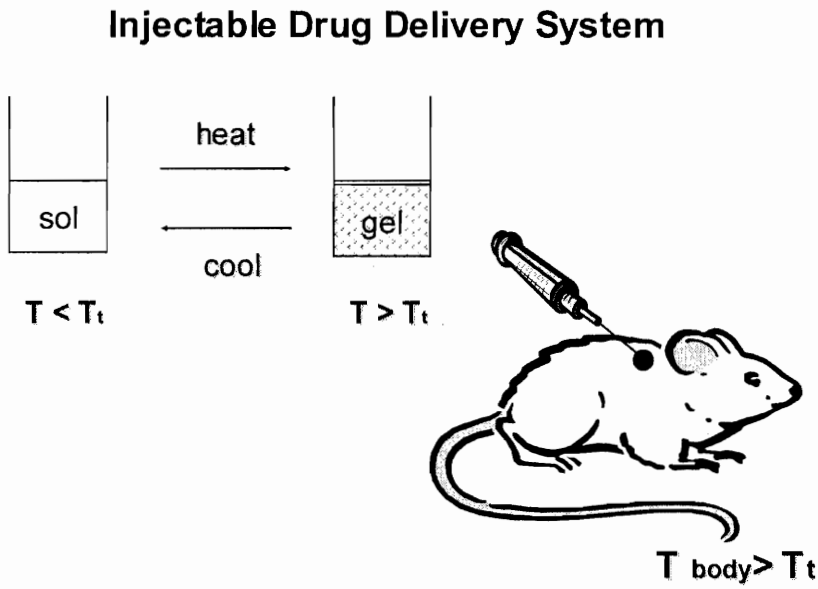
Over the last few years, there have been increasing numbers of recombinant proteins available for therapeutic applications. As a result, there have been growing numbers of publications on delivery of therapeutic proteins or peptides using polymers. There has been an effort to develop injectable systems. Since many biomedical polymers for pharmaceutical purpose are not water-soluble, only certain solvents may be used to dissolve the polymer when it is used under their toxicity limit. The concentration of the solvent in blood should be lower than the FDA limit per day basis [2]. One of the popular methods for protein or peptide delivery is to entrap those in biodegradable polymers to achieve sustained and controlled release rates [3]. Active drug substances will be released from the formulation via the combination of diffusion through the polymeric matrix and degradation. Most of sustained release formulations are designed using biodegradable polymers. The majority of biodegradable polymers are specifically based on the use of polyesters, such as poly-lactides (PLAs) and poly (lactide-co-glycolide) (PLGA) copolymers. The most representative example of biodegradable polymers used in drug delivery is poly (lactic-co-glycolic acid). Once injected in the body, those polymers undergo nonenzymatic hydrolytic

cleavage of the ester linkages to form lactic acid and glycolic acid. Lactic and glycolic acid are normal metabolic compounds which can be eliminated via the tri-carboxylic acid cycle as carbon dioxide and water [4].

As mentioned earlier, these polymers are not generally water soluble. Therefore, fabrication of protein-loaded implants or microspheres using such polymers requires use of organic solvent. The organic solvent used in the formulation should be biocompatible, water miscible and easily excreted from the body. Loaded protein or peptide drugs may lose their biological activity. Also, it maybe not possible to maintain the original conformation and structural integrity of protein and peptide therapeutics due to harsh preparation conditions and use of the organic solvents. One way to overcome these problems is to use polymer hydrogels formed by sol-gel transition in water [5], such as Poloxamers (PEO-PPO-PEO). However, such nonbiodegradable polymers exhibit toxicity *in vivo*. Therefore, biodegradable thermosensitive polymers which exhibit sol-gel transition in aqueous solution are particularly useful. The trigger of the phase transition occurs due to the thermoresponsive behavior of selected materials. For example, a low molecular weight triblock copolymer of PLGA and poly (ethylene glycol) (PEG) was designed, and a temperature-induced sol-gel transition of PEG-PLGA-PEG aqueous solution upon subcutaneous injection in rats was demonstrated [6, 7]. More recently, intratumoral injection of Oncogel^{TM} (Macromed, Inc.), an injectable formulation of PLGA-PEG-PLGA (ReGel^{TM}) and paclitaxel, demonstrated solubilization of the water-insoluble and sensitive drug and slow clearance of paclitaxel from the injection site with minimal distribution

into other organs [8]. This system is currently undergoing Phase II human clinical trials. In this study, a thermosensitive and biodegradable low-molecular weight PLGA-PEG-PLGA that exhibits sol-gel transition property in water [8] was used as a depot for the delivery of GLP-1 (Figure 2.1).

This triblock copolymer undergoes sol-gel transition above its transition temperature involving an increase in viscosity by several orders of magnitude. The polymer exhibited sol-to-gel (lower) and gel-to-sol (upper) transitions as temperature monotonically increased [9]. The lower transition is important for drug delivery applications because the solution flows freely at room temperature and becomes a gel at body temperature. The lower transition may be related to micellar growth and intra- and intermicelle phase mixing and packing, while the upper transition involves breakage of micellar structure. Critical gel concentration (CGC) and critical gel temperature are controlled by polymer parameters, such as block length and composition of the triblock copolymers, and additives, such as salts [9]. The hydrogel formed above the transition temperature possessed physical interactions between hydrophobic domains. The gel forms a controlled release drug depot for several weeks. One of advantages of using this polymer is the inherent ability to solubilize and stabilize poorly soluble and sensitive drugs, including proteins. The gel provided excellent control of the release of paclitaxel for approximately 50 days [8]. Paclitaxel loaded in the hydrophobic domain slowly diffuses out in the first phase of release followed by more rapid release when degradation of polymer matrix becomes more significant [8]. Upon subcutaneous injection, the *in situ* formed gel can maintain integrity up to several weeks.



Tt: Sol-Gel transition temperature (32°C)

Figure 2.1 Illustration of injectable drug delivery system. Above the transition temperature, the injected polymer solution with drug forms a gel.

GLP-1 is a 30 amino acid peptide hormone secreted from the intestinal L-cells in response to nutrient ingestion [10]. GLP-1 administration has been shown to reduce hyperglycemia with type 2 diabetic patients [11]. As reviewed in chapter 1, GLP-1 has a number of advantages. It makes GLP-1 a very promising candidate for type 2 diabetes treatment. GLP-1 is potent insulinotropic hormone, and its insulinotropic effect is glucose-dependent [12]. GLP-1 potentiates glucose-induced insulin secretion, but it does not have any effect on unstimulated insulin secretion. Therefore, there is no risk of hypoglycemia. It stimulates not only insulin gene transcription, but also all steps of insulin biosynthesis [13]. Therefore, insulin can be secreted continuously. GLP-1 has been shown to be capable of inducing new β-cells in the subjects with insufficient number of these cells [14]. In addition to its effect on the β-cells, GLP-1 strongly reduces glucagon secretion. Also, it inhibits gastrointestinal motility, especially gastric emptying [15]. The slower rate of gastric emptying reduces rapid postprandial increase of glucose level. All these effects render GLP-1 extremely desirable as a therapeutic agent for type 2 diabetes.

In spite of numerous advantages as diabetes treatment, there is a problem limiting the usefulness of GLP-1. The major limitation of GLP-1 as a therapeutic agent is the short half-life. GLP-1 is degraded rapidly due to the presence of a ubiquitously expressed enzyme, DPP-IV [16]. It cleaves off the two N-terminal amino acid residues. The conversion of intact, biologically active GLP-1 to its inactive metabolites occurs with an apparent half-life of 1-2 minutes [17]. More than half of released GLP-1 circulates as the truncated metabolite. It is

known that the exogenous GLP-1 is degraded by the same degradation mechanism [18]. Upon intravenous infusion of GLP-1, it is eliminated by kidneys with a half-life about 4-5 minutes [17]. After subcutaneous injection, the plasma half-life has been assessed to be approximately 60 minutes [19]. The simple subcutaneous injections of GLP-1 only lead to a small lowering of blood glucose and a short stimulation of insulin secretion. It has been shown that improved control in type 2 diabetic patients with sulfonylurea failure can be obtained by 24-hour GLP-1 exposure [20].

As a consequence of poor oral bioavailability and current lack of alternative delivery routes, GLP-1 is presently administered parentally. The plasma profile after subcutaneous injection of GLP-1 has shown a sharp peak concentration and was lowered to an effective level in 1 to 3 hours [19, 21]. Therefore, continuous infusion or multiple injections are required for clinical application of GLP-1. When once a day administration is desired, pharmacological concentrations should be maintained at least 10 hours. In addition, it is better not to have frequent initial peak plasma concentrations as seen with frequent injections [22]. Therefore, the absorption rate should be delayed. For formulation of insulin or glucagon, zinc chloride or protamine sulphate has been used to protract the absorption rate [23, 24]. Complex formation of GLP-1 with zinc will have similar effect to complexation of insulin and zinc [25].

In this study, a sustained release system of PLGA-PEG-PLGA polymer depot was used with zinc-complexed GLP-1. Zinc stabilizes GLP-1 and reduces

its solubility. This system was tested in Zucker diabetic fatty (ZDF) rats. ZDF rat is a type 2 diabetic animal model which shows impaired glucose tolerance caused by inherited obesity.

2.2 *In Vitro* Release of GLP-1 from PLGA-PEG-PLGA

2.2.1 Materials

Glucagon-like peptide-1 (7-37) (GLP-1) was synthesized at the Emory University Microchemical Facility (Atlanta, GA). Poly((DL-lactic acid-co-glycolic acid)-b-ethylene glycol-b-(DL-lactic acid-co-glycolic acid)) triblock copolymer (ReGel^{TM}) was supplied by MacroMed, Inc. (Sandy, UT). Briefly, this polymer was synthesized from PEG1450, and lactide and glycolide in the mole ratio of 4:1 (Figure 2.2). Under dry nitrogen atmosphere polyethylene glycol (60 g) was dried in a three-necked flask under vacuum (1–2 Torr) and stirring at 150°C for 3 hours. Lactide and glycolide were added in the mole ratio of 4:1, respectively, and the reaction mixture was heated under vacuum for 30 minutes. After all the lactide and glycolide were melted, 0.04 g of stannous 2-ethylhexanoate were added and the reaction mixture was further heated at 155°C for 8 hours. Bath temperature was reduced to 150°C and unreacted monomers were removed under vacuum. Crude polymer was dissolved in cold water (5–8°C). After complete dissolution, the polymer solution was heated to 80°C to effect precipitation of the polymer and to remove water-soluble low-molecular weight polymer and unreacted monomer. Precipitated polymer was isolated by decanting the supernatant and re-dissolving in ice-cold water. Heating, precipitation and decantation was

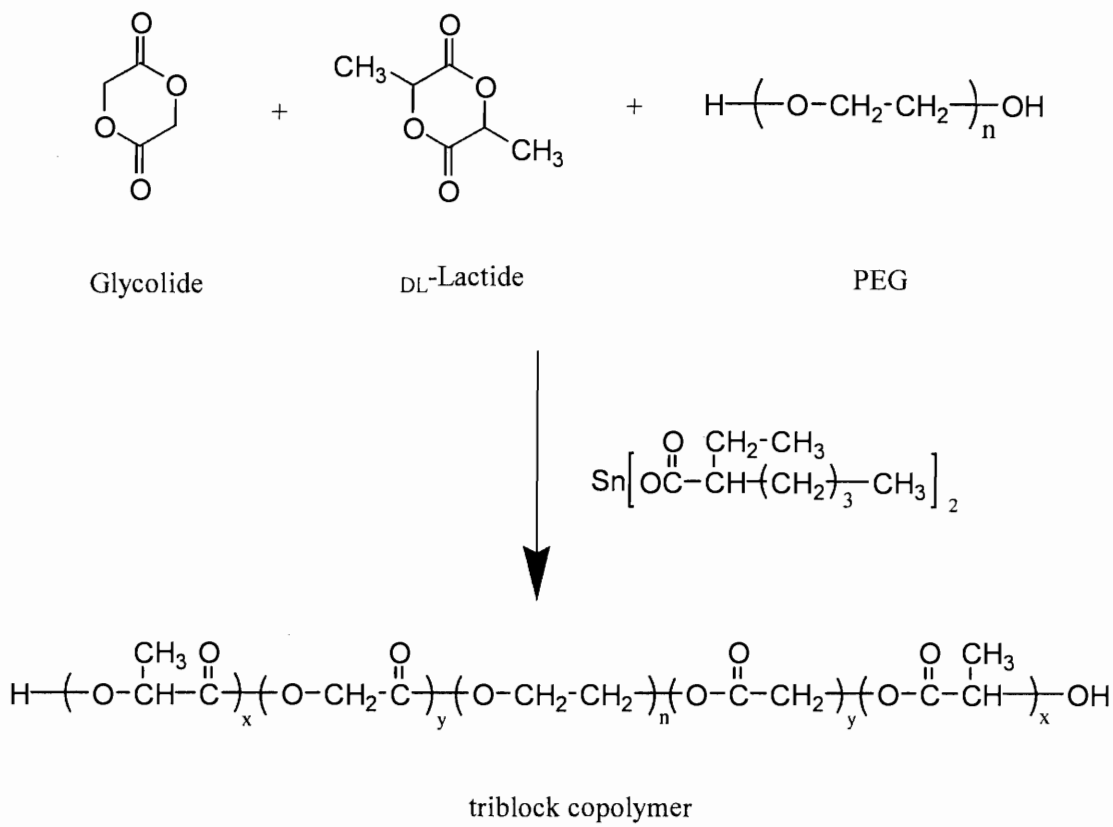


Figure 2.2 Synthesis scheme of PLGA-PEG-PLGA triblock copolymer.

repeated once again and purified polymer was obtained. Residual water was removed by freeze drying to give triblock copolymer as a viscous semisolid in ~85% yield. Residual lactide and glycolide monomer levels were analyzed by HPLC and shown much less 0.5% (w/w).

2.2.2 Experimental Methods

The PLGA-PEG-PLGA triblock copolymer was dissolved in 30 mM phosphate buffer, pH 7.4 at room temperature to make a 23 % solution. For the first group, GLP-1 was dissolved in 1mM HCl at concentration 5 mg/mL and pH adjusted to 7.0 with 0.1 M NaOH. The formed suspension was incubated at 4 ºC overnight, and mixed with PLGA-PEG-PLGA triblock copolymer solution prepared as described above. To select the optimal concentration of ZnGLP-1 in polymer solution, three different concentrations (5mg/mL, 10 mg/mL, and 20 mg/mL) were tested. For the second group, the same amount of GLP-1 was precipitated in the presence of zinc prior to loading essentially as described by Gappa et al. [26]. After GLP-1 dissolution, zinc chloride was added (final pH was ~3.5). Then, pH was adjusted to 7.0 with 0.1 M NaOH. The GLP-1 solution was mixed with PLGA-PEG-PLGA triblock copolymer solution as described above.

Next, 1 ml of each formulation was placed in vials, incubated at 37 °C until gels formed, and 20 ml of 10 mM PBS, pH 7.4, solution was added as release medium. Samples were withdrawn from the release medium and PBS was replaced daily. Samples were analyzed by reversed-phase high performance liquid chromatography (RP-HPLC) to measure the concentration of GLP-1.

Shimadzu SCL-10Avp liquid chromatograph was equipped with a C_4 column (Vydac), which was previously equilibrated. The mobile phases were water and acetonitrile containing 0.1 % trifluoro acetic acid (TFA) with a gradient 2% B/min and the flow rate was 1.2 ml/min.

Data was analyzed by two-way ANOVA for repeated measures using a generalized linear model. In all cases a p value of <0.05 was considered to be statistically significant.

2.2.3 Results and Discussion

The concentration of ZnGLP-1 in polymer solution for this study was determined as a result of dose dependency study. The amount of released GLP-1 was measured by reversed-phase HPLC. Figure 2.3 shows the result from the HPLC analysis performed on the release media. The analysis shows a sharp, clean peak at the retention time of 14.9 minutes, which is expected for GLP-1.

To select the optimal concentration of ZnGLP-1 in the polymer solution for the *in vivo* study, 5, 10, and 20 mg of ZnGLP-1 was incorporated to 1 mL of polymer solution. Figure 2.4 shows the daily amount of released GLP-1 for each loading amount. When the amount of GLP-1 was increased from 5 mg to 10 mg, the amount of daily release was increased significantly over the period of 15 days and showed similar release patterns. However, when the loaded GLP-1 was increased from 10 mg to 20 mg, the initial release for the first day was greater in the case of 20 mg loading, yet the released amount after the initial release phase was not statistically significantly different. This can be attributed to the domain

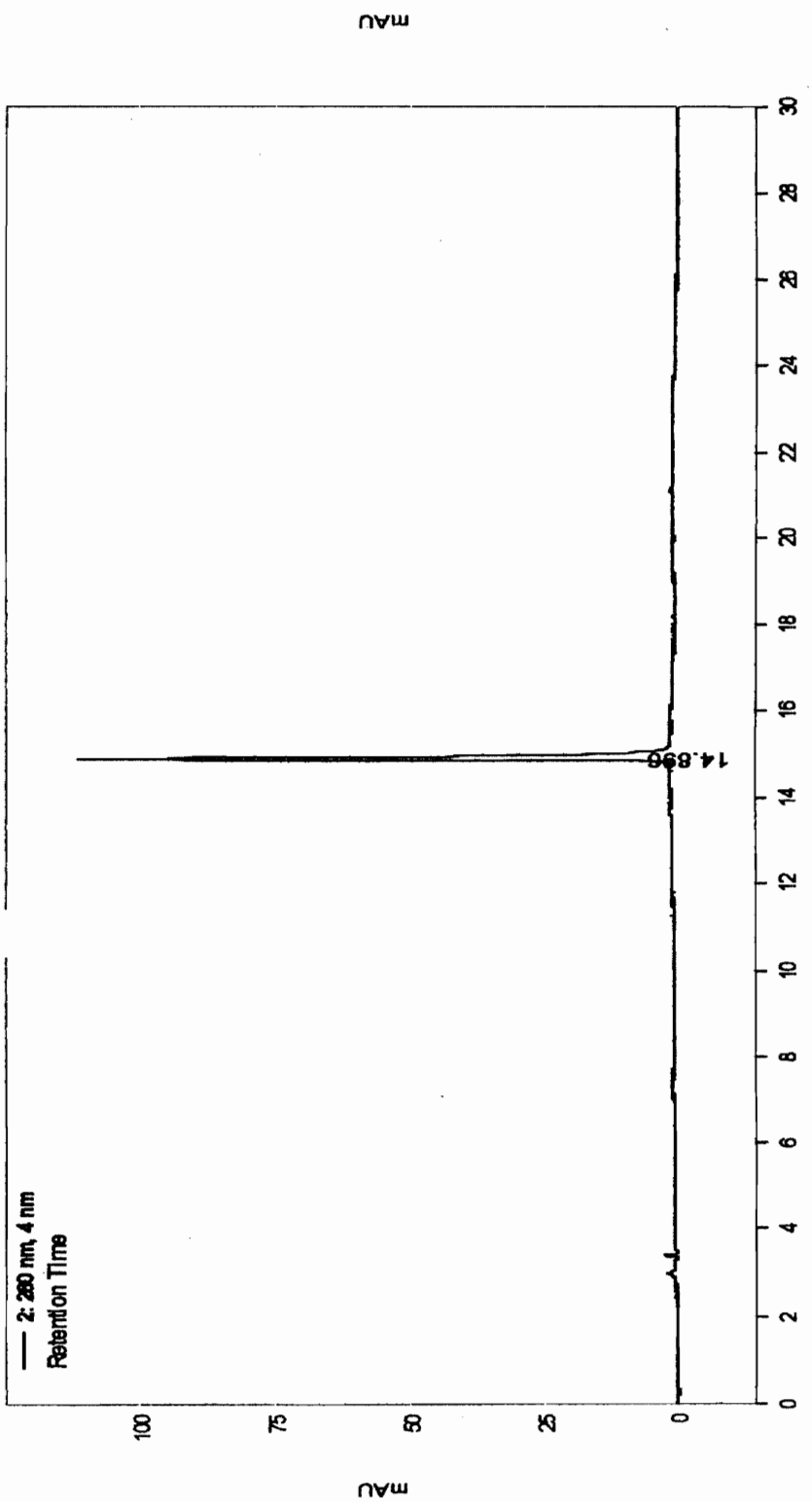


Figure 2.3 RP-HPLC

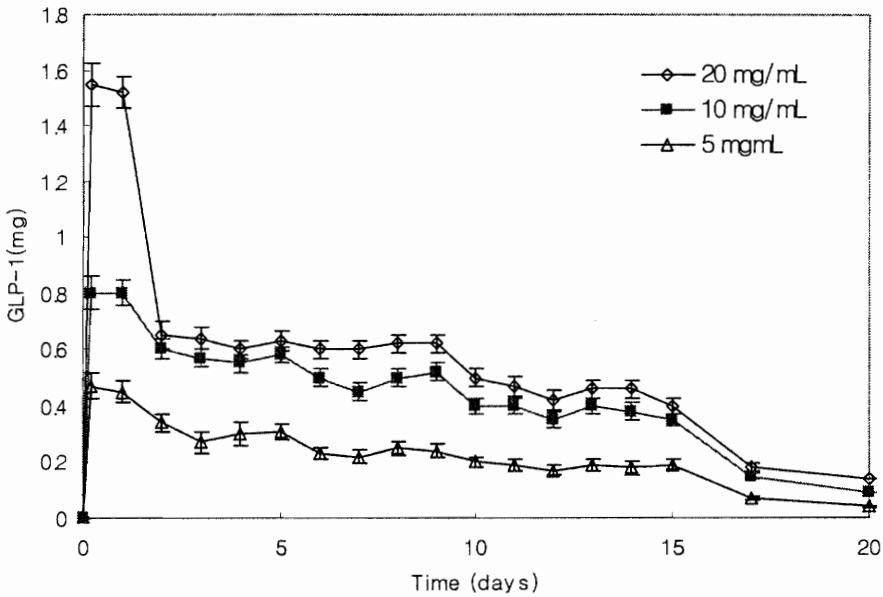


Figure 2.4 Daily amount of released GLP-1 from PLGA-PEG-PLGA formulation *in vitro.* Released amount of GLP-1 from 20 mg GLP-1/ PLGA-PEG-PLGA (open circle), 10 mg GLP-1/ PLGA-PEG-PLGA (closed square), and 5 mg of GLP-1/ PLGA-PEG-PLGA (open triangle) was measured for the indicated times. The graph represents the average ± SEM, n=3.

structure of the triblock copolymer hydrogel: hydrophilic domains and hydrophobic domains throughout the gel matrix in which the hydrophobic domains serve as physical crosslinks. When the drug loading is increased, the hydrophobic domains are saturated and the rest should remain in the hydrophilic domain. Drug loaded in the hydrophilic domain is responsible for initial release phase, which is more rapid, whereas drug loaded in the hydrophobic domain is responsible for slower release in the later phase. For this reason, it is possible that a larger amount of 20 mg of GLP-1 per gram of gel may have been subject to initial release compared to 10 mg loading.

Increasing the loading from 10 mg to 20 mg of GLP-1 in the gel can produce the benefit of increase in released amount only for the first day which is not beneficial for the purpose of achieving sustained effect of GLP-1 for two weeks whereas increasing the loading from 5 mg to 10 mg showed more release over the whole duration of interest. Therefore, GLP-1 loading of 10 mg was chosen for further *in vivo* study. The *in vitro* release of GLP-1 from the PLGA-PEG-PLGA polymer depot was investigated and the data are presented in Figure 2.5. The graph shows the % cumulative amount of released GLP-1 over the period of 20 days. The release of GLP-1 from the triblock copolymer hydrogel without using zinc complex shows a big burst with more than 60 % of initial loading released in day 1 and 80 % released in 3 days. As compared to the first group, the release of zinc-complexed GLP-1 exhibited no initial burst and displayed constant rate and an almost linear release profile over 2 weeks to reach almost 90 % of the initial loaded amount.

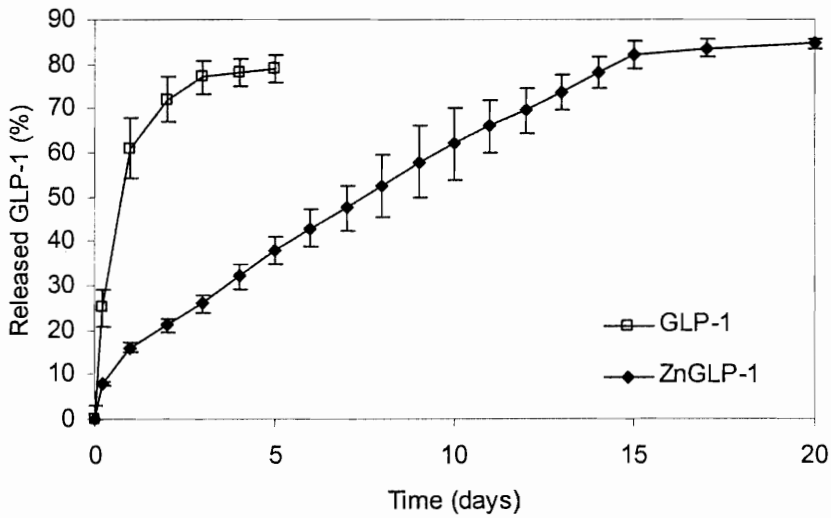


Figure 2.5 Cumulative amount of released GLP-1 from PLGA-PEG-PLGA formulation. Released amount of GLP-1 from GLP-1/ PLGA-PEG-PLGA (open circle) and ZnGLP-1/ PLGA-PEG-PLGA (closed square) was measured for the indicated times. The graph represents the average ± SEM, n=3.

The thermosensitive, biodegradable triblock copolymer (ABA-type triblock copolymer composed of PLGA and PEG) in aqueous solution (ReGel^{TM}) is a free-flowing sol at room temperature and becomes a gel at body temperature. Therefore, polymer forms a water-insoluble gel once injected. This hydrogel formed above transition temperature possessed physical interactions between hydrophobic domains (Figure 2.6).

Dynamic mechanical analysis revealed that an increase in viscosity of approximately four orders of magnitude accompanies the sol-gel transition. The gel forms a controlled release drug depot with delivery times ranging from 1 to 6 weeks [8].

The first study that demonstrated the feasibility of using this system as a carrier for controlled release of protein was performed with insulin [27]. No initial burst and a constant release of human insulin from the polymer depot *in vitro* was observed due to the domain structure of the gel and modification of the association states of insulin by zinc. Animal studies using Zucker diabetic fatty rats were performed to verify, *in vivo*, the release profile of insulin from this ABA block copolymer hydrogel. The polymer formulation maintained insulin release up to 2 weeks, which could allow diabetic patients to reduce the number of insulin injection twice a month for basal insulin requirements [28]. In this study we have shown that this system can also be used as a drug delivery carrier for the continuous release of GLP-1. The stimulating effect of zinc-complexed GLP-1 on the secretion of insulin from pancreatic islets was demonstrated by Gappa *et al.* [26].

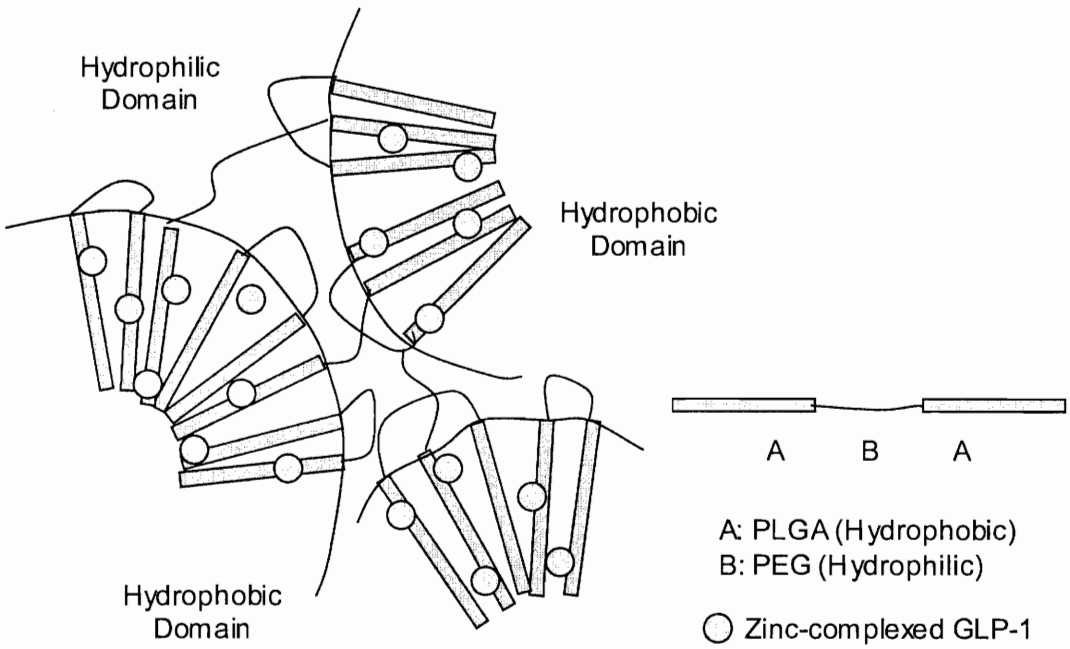


Figure 2.6 Physical crosslinks in the PLGA-PEG-PLGA in water showing hydrophobic domain structures.

The release of zinc-complexed GLP-1 from PLGA-PEG-PLGA triblock copolymer showed no initial burst and constant release rate as demonstrated by *in vitro* release study. It is evident that zinc complexation provided a means to stabilize the peptide physically and chemically, and to achieve dramatic reduction of initial burst as compared to GLP-1 release from the same hydrogel without zinc. Uncomplexed GLP-1 is highly soluble in water and thus, the majority GLP-1 stays in the hydrophilic domain of the PLGA-PEG-PLGA triblock copolymer, and is quickly released via water channels, exhibiting burst release in 3 days.

2.3 *In Vivo* Release of GLP-1 from PLGA-PEG-PLGA

2.3.1 Materials

Twenty male Zucker Diabetic Fatty (ZDF) rats (410-460 g) were purchased from Charles River Laboratories (Wilmington, MA) at 11 weeks of age. All animals used in this study were housed according to the principles established for care and use of laboratory animals (21-23°C, 12-12 h light-dark cycle). They were fed *ad libitum* with Purina 5008 (6.5% fat). The experimental protocols concerning the use of laboratory animals were reviewed and approved by the Institutional Animal Care and Use Committee of University of Utah.
The ZDF is an inbred rat model through both genetic mutation and a managed diet. It is possible to mimic human adult onset diabetes and its related complications, when male ZDF rats are fed a diet of Purina 5008, which contains high fat (6.5%). Since the ZDF rat shows impaired glucose tolerance caused by the inherited obesity, it is a model for type 2 diabetes [29, 30]. The obese male

ZDF rat is considered to be a good model for type 2 diabetes and has been used since the late 1980s [30].

2.3.2 Experimental Methods

The experimental animals, male ZDF rats (12 weeks), were divided into two groups of six animals each. Both groups were fasted overnight and anesthesia was induced by intramuscular injection of Pentobarbital (60 mg/kg). The first group was injected with 1 ml of blank triblock copolymer aqueous solution. The second group was administrated subcutaneously 1ml formulation containing 10 mg ZnGLP-1. The dose was estimated according to intravenous infusion data. The concentration of GLP-1 in polymer solution was selected as a result of a dose dependency study. At designated times (day 1, 2, 3, 4, 5, 6, 7, 9, 11, 13, and 15), 600 μl of blood samples were obtained from the tail vein of ZDF rats.

2.3.2.1 Assay of GLP-1, Insulin, and Glucose Level

GLP-1 concentration was determined by an ELISA kit (Linco Research) that measures biologically active GLP-1 forms in the sample. The samples were treated with dipeptidyl peptidase IV inhibitor (Linco Research). The serum insulin levels were determined by using insulin radioimmunoassays kit (ICN Pharmaceuticals, Costa Mesa, CA). The blood glucose levels of the ZDF rats were monitored using Accucheck-Instant (Boehringer Mannheim, Indianapolis, IN).

2.3.2.2 Glucose Tolerance Test

Two groups of rats (n=3) were subjected to an intraperitoneal glucose tolerance test (IPGTT) at day 7. Rats were fasted for 15 hours. A basal blood sample was collected from a tail vein (t=0). The rats were injected with glucose (1.5 g / kg) intraperitoneally and additional blood samples were collected at t = 30, 60, 120, and 180 minutes. Blood glucose levels were measured by using Accucheck-Instant (Boehringer Mannheim, Indianapolis, IN).

2.3.2.3 Statistical Analysis

The statistical program, SPSS for Windows (SPSS, Chicago, IL) was used for statistical analysis. Data for the effects of GLP-1 in the ZDF rats were analyzed by a general linear model. In all cases P values of less than 0.05 were taken as statistically significant. All data are presented as means ± SEM.

2.3.3 Results and Discussion

An animal study using ZDF rats was performed with ZnGLP-1 / PLGA-PEG-PLGA triblock copolymer formulation. The polymer solution with ZnGLP-1 formed a gel once injected, and its integrity was maintained for about a week. Figure 2.7 shows the formed gel at 7^{th} day after subcutaneous injection of polymer solution with ZnGLP-1. Figure 2.8 shows the plasma concentration of GLP-1 after subcutaneous injection as determined by ELISA. It should be noted that the detection is limited only to biologically active form of GLP-1. After injection of the formulation, GLP-1 concentration in plasma reached 200 ng/L

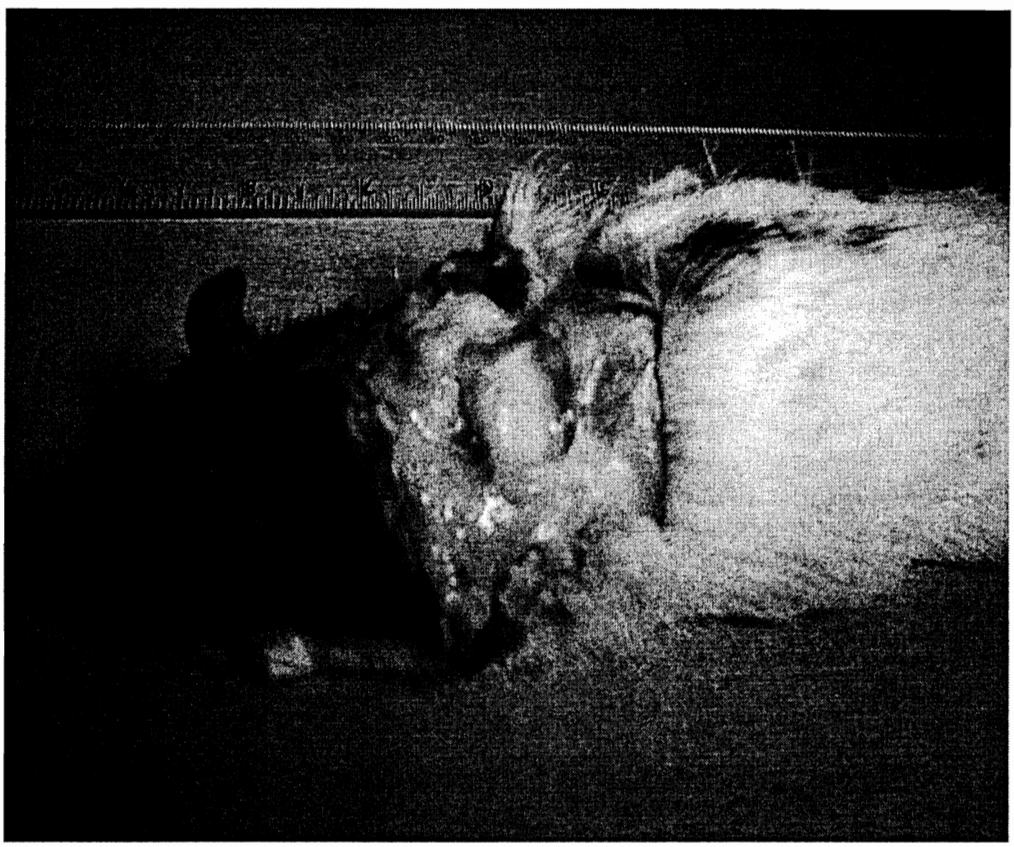


Figure 2.7 The formed gel at 7th day after subcutaneous injection of polymer solution with ZnGLP-1.

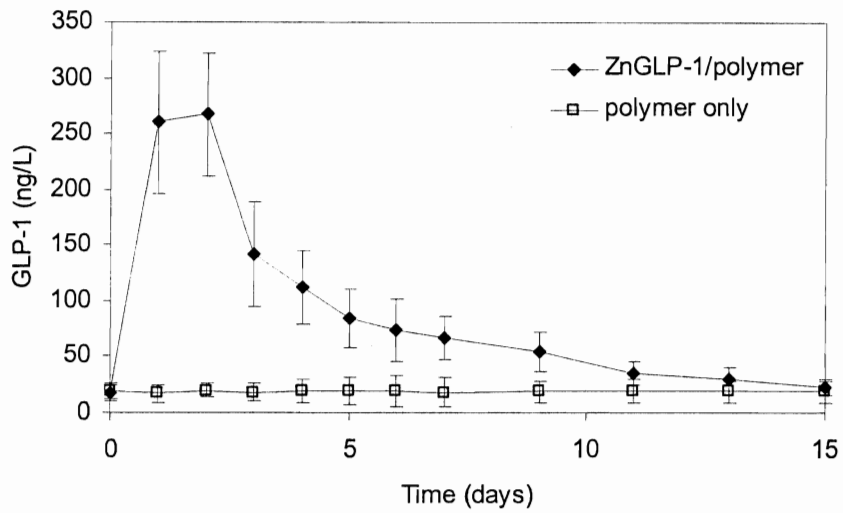


Figure 2.8 Plasma GLP-1 levels in rats treated with ZnGLP-1/PLGA-PEG-PLGA formulation (closed circle) and PLGA-PEG-PLGA (open square) were assayed for the indicated times. The graph represents the average ± SEM, each group was composed of six rats. Statistical significance between groups (PLGA-PEG-PLGA vs. ZnGLP-1/PLGA-PEG-PLGA) was determined by repeated-measures ANOVA: $P < 0.05$.

initially (day 1 and day 2) and later significantly higher GLP-1 levels (> 50 ng/L) were maintained compared to that of the control group (treated with blank polymer) with duration of at least 14 days. Figure 2.9 shows the insulin levels in the plasma samples over 15 days. Injection of blank polymer hydrogel shows the constant basal insulin levels of ~ 1.8 ug/mL. The injection of zinc-complexed GLP-1/ PLGA-PEG-PLGA triblock copolymer induced increased insulin levels about 3 ug/mL initially which were maintained over several days with subsequent gradual decrease over 15 days. The significant difference between control and positive groups was found up to day 11.

Blood glucose profile as a result of GLP-1 release is depicted in Figure 2.10. While the injection of blank polymer resulted in no impact on the blood glucose level that was maintained at 350~400 mg/dL, GLP-1/ PLGA-PEG-PLGA triblock copolymer demonstrated significant glucose lowering activity over the 15-day period in type 2 diabetic animals. Also, the change in blood glucose level over time is consistent with the plasma insulin profile (Figure 2.9).

Blood glucose profile after intraperitoneal glucose administration is shown in Figure 2.11. Giving 1.5 g / kg glucose evoked pronounced glucose excursions in both groups of animals. However, blood glucose excursion was significantly lower in GLP-1/ PLGA-PEG-PLGA triblock copolymer treated rats following intraperitoneal glucose loading. At day 7, the plasma GLP-1 levels of GLP-1/ PLGA-PEG-PLGA triblock copolymer treated rats were maintained at higher level with value about 60 ng/L (compared with 20 ng/L in control animals). This figure clearly shows that released GLP-1 was able to significantly improve glucose

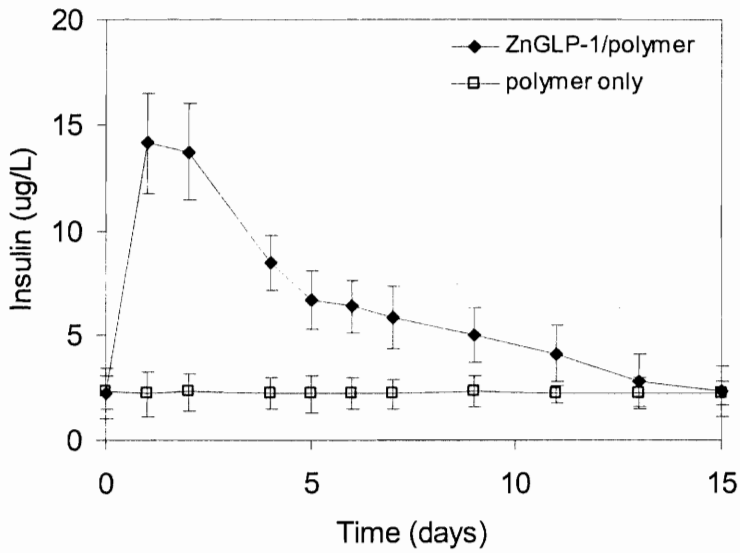


Figure 2.9 Plasma insulin levels in rats treated with ZnGLP-1/PLGA-PEG-PLGA formulation (closed circle) and PLGA-PEG-PLGA (open square) were assayed for the indicated times. The graph represents the average ± SEM, each group was composed of six rats. Statistical significance between groups (PLGA-PEG-PLGA vs. ZnGLP-1/PLGA-PEG-PLGA) was determined by repeated-measures ANOVA: $P < 0.05$.

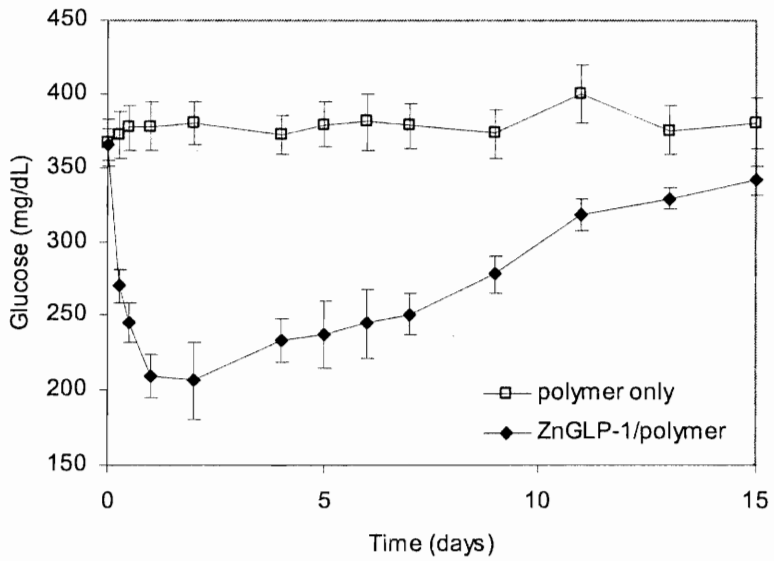


Figure 2.10 Blood glucose levels in rats treated with ZnGLP-1/PLGA-PEG-PLGA formulation (closed circle) and PLGA-PEG-PLGA (open square) were assayed for the indicated times. The graph represents the average ± SEM, each group was composed of six rats. Statistical significance between groups (PLGA-PEG-PLGA vs. ZnGLP-1/PLGA-PEG-PLGA) was determined by repeated-measures ANOVA: $P < 0.05$.

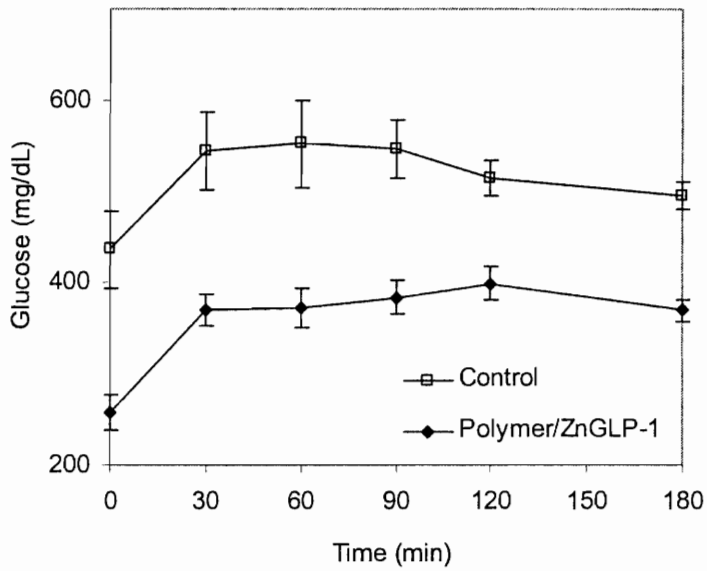


Figure 2.11 Blood glucose level in ZDF rats after glucose challenge (IPGTT). At day 7, blood glucose levels in rats treated with ZnGLP-1/PLGA-PEG-PLGA formulation (closed circle) and PLGA-PEG-PLGA (open square) were assayed for the indicated times after intraperitoneal glucose injection. The graph represents the average ± SEM, each group was composed of five rats. Statistical significance between groups (PLGA-PEG-PLGA vs. ZnGLP-1/PLGA-PEG-PLGA) was determined by repeated-measures ANOVA: $P < 0.05$.

tolerance in the animals as early as 30 minutes after intraperitoneal glucose injection.

Finally, subcutaneous injection of GLP-1/ PLGA-PEG-PLGA triblock copolymer was well tolerated, and there was no sign of chronic inflammation around the injection sites during the experiment.

The present results show that the elevated blood glucose level in type 2 diabetic rats can be controlled by a single injection of PLGA-PEG-PLGA triblock copolymer formulation with zinc-complexed GLP-1 for 2 weeks. The clinical application of GLP-1 has been limited by its extremely short half-life of 2-3 min [31]. Although many practical problems still remain to be solved, a therapy based on the GLP-1 is one of the most promising recent approaches to the treatment of type 2 diabetes. A number of new approaches are currently under investigation for using GLP-1 as therapeutic agent [32-34]. To overcome its instability, different analogs of GLP-1 [33, 35] and inhibitors of dipeptidyl peptidase IV [36] are currently tested in clinical trials.

Even though the use of DPP IV-resistant analogs of GLP-1 shows considerable promise, it still requires at least everyday injection to achieve therapeutic effect. Moreover, a complete normalization of glucose concentrations has only been demonstrated with the intravenous infusion of native GLP-1 [11, 20, 31].

Early stage of type 2 diabetes is characterized by a progressive loss of first phase insulin secretion and a compensatory increased second phase secretion [32, 33]. Delayed insulin secretion after meal ingestion causes

postprandial hyperglycemia [34]. Continuous release of GLP-1 can reduce postprandial glycemic excursions effectively. Due to its glucose-depending insulinotropic effects, GLP-1 actions do not cause hypoglycemia [11]. Although GLP-1 is a very attractive therapeutic agent for type 2 diabetes, GLP-1 is very unstable in the blood stream, requiring continuous infusion or frequent subcutaneous injections. Thus, the use of PLGA-PEG-PLGA triblock copolymer as a controlled release depot for GLP-1 in type 2 diabetic patients will result in improved patient compliance.

Animal studies using ZDF rats have been performed to study the bioactivity of the released GLP-1. The ZDF rats used were of 12 weeks of age with fully developed type 2 diabetes. Hyperglycemia initially manifested at about 7 weeks of age, and all obese male rats fed special chow are fully diabetic by 12 weeks [30]. As shown in Figure 2.8, the plasma GLP-1 levels were maintained *in vivo* for about two weeks at concentrations significantly higher than that of control group. This was manifested in increased plasma insulin and decreased blood glucose levels over the same time period. In Figure 2.9, insulin level reached and stayed at higher levels over 2 weeks as long as there was significantly increased GLP-1 levels in plasma. This means that the continuously released GLP-1 from the subcutaneously injected PLGA-PEG-PLGA triblock copolymer demonstrated an insulinotropic effect. Blood glucose level was accordingly maintained to the near normalized level as shown in Figure 2.10. The glucose level dropped to the level significantly lower (~200 mg/dL) than the control (~400 mg/dL) as long as the insulin level was maintained. In contrast, the control group in Figure 2.4

shows endogenous insulin levels yet this has no effect on lowering blood glucose, signifying insulin resistance that is characteristic of the type 2 diabetes. The IPGTT data suggested that released GLP-1 was able to significantly improve glucose tolerance. The GLP-1 treated animals had much lower peak glucose levels in response to the glucose challenge, with the values peaking at 390 mg/dL (compared with 550 mg/dL in control animals); thus, the GLP-1 delivery system improved glucose tolerance and hyperglycemia.

2.4 Conclusions

As shown by the data, a steady amount of GLP-1 was released from PLGA-PEG-PLGA triblock copolymer formulation depot more than ten days after a single subcutaneous injection. It is evident that the GLP-1 released from the thermosensitive biodegradable hydrogel (ReGel^{TM}) formulation is bioactive as it stimulates insulin secretion *in vivo*. Also, GLP-1 released from PLGA-PEG-PLGA triblock copolymer formulation depot results in the improved glucose tolerance. Therefore, it can be concluded that it is feasible to use PLGA-PEG-PLGA triblock copolymer formulation with zinc-complexed GLP-1 as a 2 week delivery system, making it a twice-a-month injection depot. Dose may be further adjusted by the loaded amount of GLP-1 and gel concentration for normalizing blood glucose levels completely.

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CHAPTER 3

DESIGN OF GLUCAGON-LIKE PEPTIDE-1 PLASMID AND *IN VITRO* STUDY

3.1 Introduction

Glucagon-like peptide-1 (GLP-1) is a 30 amino acid hormone produced by intestinal L-cells. It has been proposed that GLP-1 can be used as a new treatment for type 2 diabetes mellitus because it acts to augment insulin secretion and its effectiveness is maintained in type 2 diabetic patients. In spite of its many remarkable advantages as a therapeutic agent for diabetes, GLP-1 is not immediately clinically applicable because of its extremely short half-life [1]. One way to overcome this drawback is GLP-1 gene delivery which enables GLP-1 production in the body. The plasmid containing GLP-1 minigene was tested for *in vitro* transfection of mouse insulinoma cells [2]. A DNA fragment of the human glucagon gene encoding for GLP-1 was used for plasmid construction. GLP-1 plasmid with GLP-1 cDNA was also tested *in vivo* as well as *in vitro* [3]. In that study, the expression level of GLP-1 was not high enough for normalizing blood glucose levels. The main aim of this study was to design an effective GLP-1 delivery system for therapeutic use for type 2 diabetes treatment based upon

construction of an efficient GLP-1 plasmid and its delivery.

Gene therapy can be divided into two components. One is a gene expression system which encodes a specific protein, and the other is a delivery system which controls the delivery of plasmid for gene expression. In this chapter, the gene expression system was studied for GLP-1 gene therapy. To make a gene transfer vector more efficient, expression efficiency should be enhanced. The expression levels of the introduced gene depend mainly on the strength of transcriptional regulatory elements and the transduction efficiency of the gene transfer vector [4]. For high-level expression, one important aspect is the optimization of the plasmid vector. By using optimal transcription regulatory elements, it will be possible to enhance expression efficiency. It is desirable to use strong promoters in order to obtain a high level of transcription and expression of the gene. Although various kinds of transcriptional regulatory elements have been tried, only several elements are considered ubiquitously strong. The examples of suitable promoters for expression of genes in animal cells include the beta-actin promoter, cytomegalovirus (CMV) promoter, and the Rous Sarcoma Virus (RSV) LTR-promoter [5]. Enhancer sequences are a set of eucaryotic DNA elements that increase transcriptional efficiency in a manner independent of their position and orientation. The physical boundaries between promoter and enhancer elements are not clear [4]. Most genes from higher eukaryotes contain introns which are removed during RNA processing. It has been shown that the inclusion of introns can lead to more efficient expression than identical constructs lacking introns [6]. Also, most eukaryotic mRNAs have a

poly(A) tail at their 3' ends, which is added during a complex process that involves cleavage of the primary transcript and a coupled polyadenylation reaction [4]. The poly(A) tail is important for mRNA stability and translatability [7].

Plasmid regulatory elements consist of promoter, enhancer, intron and polyadenylation signal. Since it was found that the effect of different introns and polyadenylation signal on expression levels is relatively small, selection of strong promoters/enhancers was focus of the first approach. Among many viral promoters/enhancers, the cytomegalovirus (CMV) promoter/enhancer has been found to be one of the strongest ones. Simian virus 40 (SV40) early region promoter/enhancer has been used as a viral promoter and has shown very high levels of expression in muscle, liver, and pancreas. As a cellular promoter, chicken β-actin promoter showed higher expression levels than the CMV promoter in mouse liver [3].

The other approach to enhance expression efficiency is to increase nuclear transport of plasmids into cells. It is known that nuclear transport of plasmids in mammalian cells is the main barrier in nonviral gene delivery [8]. After release from endosome, the therapeutic plasmid should enter the nucleus after which mRNA and protein can be subsequently produced. However, most plasmids are too large to diffuse through the nuclear pore complex (NPC) [9]. To enter the nucleus, exogenous gene must either contain nuclear localization signals (NLSs) or form oligomers with other proteins that contain an NLS [10].

Transcription factors are proteins known to contain NLSs. These transcription factors could be utilized as carrier proteins to import DNA to the

nucleus. Since transcription factors are known to bind to specific DNA sequences, if such DNA sequences are present in the cytoplasm, they can form a complex with these proteins. The NLSs present in this complex can then interact with the NLS receptor and enter the nucleus by the conventional nuclear protein import machinery [11]. Nuclear factor κB is a family of transcription factors present in every cell type [12]. NFκB transcription factors are heterodimers or homodimers of a family of related proteins characterized by the Rel homology domain (RHD).

They form two subfamilies, one with activation domains (p65, RELB, and c-REL) and the other without activation domain (p50, p52) [13]. The prototypical NFκB is a heterodimer of p65 and p50. In the absence of stimulus, most of NFκB is present in the cytoplasm in an inactive state in a complex with inhibitor protein IκB. IκB is a family of seven inhibitory subunits which can be characterized by multiple ankyrin-like repeats [14]. There are three IκB proteins, IκB-α, IκB-β, and IκB-ε, that have been identified. Among these IκB proteins, IκB-α is the most abundant inhibitory protein for NFκB [15]. All IkBs contain multiple copies of a 30-33 amino acid sequence, ankyrin repeats, which mediate the association between IκB and NFκB dimers. The ankyrin repeats interact with a conserved N-terminal region, called the Rel homology domain of NFκB proteins. As a result of masking of the NLS, nuclear translocation is prevented. Signals that induce NFκB activity cause the phosphorylation of IκBs, their dissociation and subsequent degradation, allowing NFκB proteins to enter the nucleus and induce gene expression [12]. IκB serves as a cytoplasmic anchor for NFκB, hence when IκB is degraded, NFκB can migrate into the nucleus. NFκB is able to carry

messages into nucleus because it communicates with both the cytoplasm and the nucleus. However, without any stimuli to induce NFκB activity, there are active NFκB molecules in cytoplasm due to their low-level migration into nucleus. In one study, under nonstimulatory conditions, transfection was enhanced by 2.6- to 5.8- fold by incorporating NFκB binding sites into plasmids [16]. Since nuclear factor κB contains a NLS, it will allow nuclear entry of plasmid by a piggyback mechanism. By incorporating nuclear factor κB binding sites in the constructed GLP-1 plasmids, it may be possible to increase the import of plasmid DNA into nuclei (Figure 3.1). In addition, it has been suggested that the activation of NFκB participates in chronic disorders including diabetes [17]. If cells are exposed to stressful stimuli, IκB will be rapidly phosphorylated and degraded. Thus, NFκB can translocate into the nucleus [18].

In this study, GLP-1 plasmid was designed to increase expression level and tested *in vitro*. Since gene expression level is largely regulated by the level of transcription, the first part of GLP-1 plasmid design was based on the optimization of transcriptional regulatory elements for higher gene expression. The strength of each promoter/enhancer has been tested indirectly by monitoring the GLP-1 gene activity following introduction into the cell via transfection of the plasmid/carrier complex into the cells. The second part of GLP-1 plasmid design was based on increasing nuclear transport which is the main barrier in non-viral gene delivery. By incorporating a nuclear factor κB binding site to the constructed GLP-1 plasmids, the expression level can be increased due to the enhancement of nuclear import of plasmid DNA. After *in vitro* transfection studies, the GLP-1

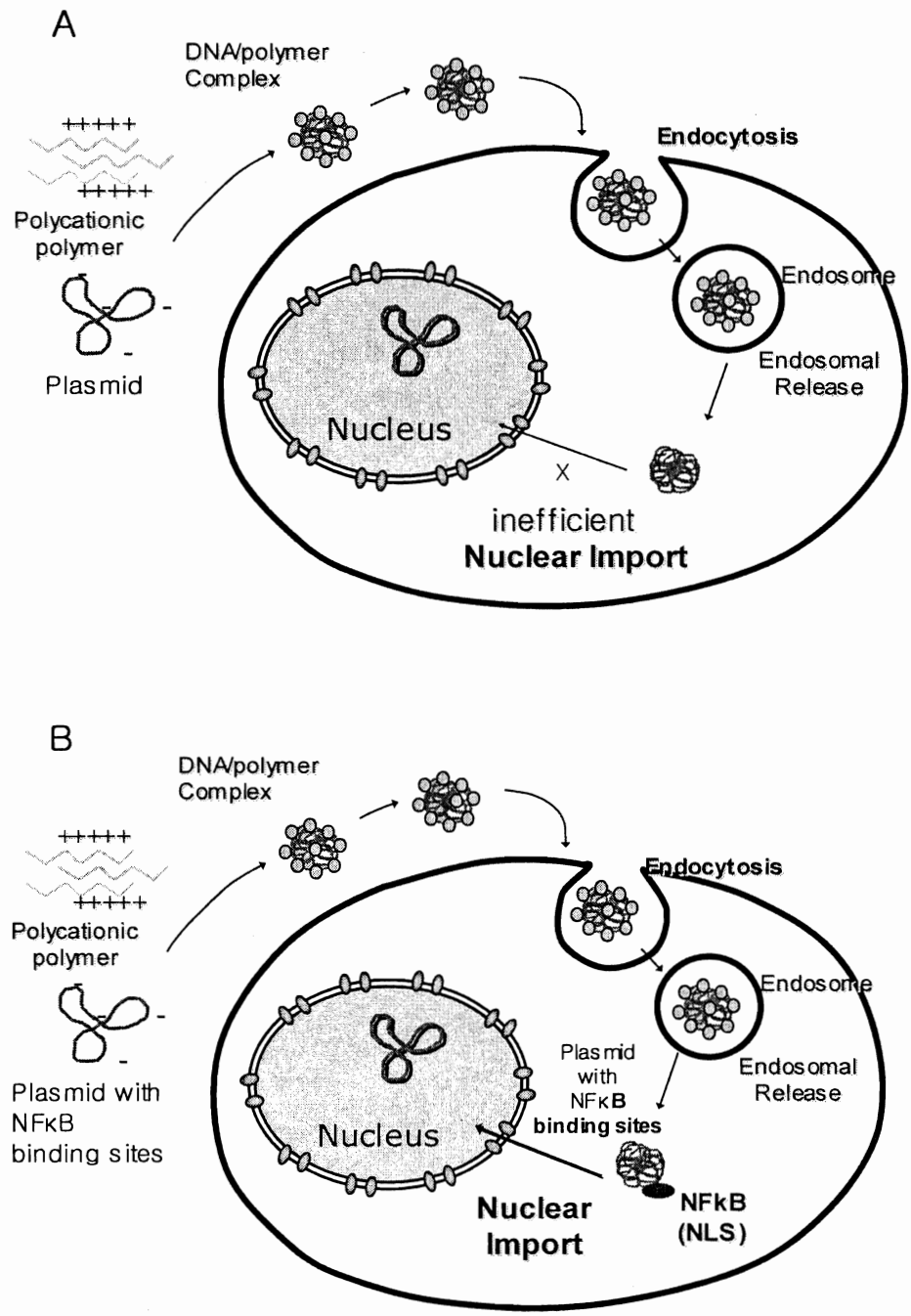

Figure 3.1. A. Inefficient uptake of DNA into nucleus, B. Uptake of DNA is enhanced due to the presence of a specific DNA sequence, a NFκB binding site, in the plasmid. NFκB binds to the plasmid, and carry it into the nucleus due to the presence of a NLS in NFκB.

plasmid with the maximal expression level was selected. For a gene delivery carrier, polyethylenimine (PEI) was used.

Although viral gene carriers can transfer gene more effectively, they have disadvantages, compared to nonviral gene carriers. Nonviral gene transfer agents have several advantages over recombinant viruses. They are noninfectious, less immunogenic, less cytotoxic, easy to handle, and reproducible. Also, they can accommodate a large DNA plasmid and be modified for specific cell targeting. Cationic polymers have been used as non-viral gene carrier. The cationic polymer forms complexes with plasmid by electrostatic interactions. Polymeric gene carriers can protect plasmid against enzymatic degradation. As reviewed in Chapter 1, PEI has been commonly used as gene carrier. PEI is synthesized by acid-catalyzed, ring-opening polymerization of aziridine. It has been widely used because its ability of complex formation with DNA within physiological pH range and endosomal escape [19]. PEI has shown relatively high transfection efficiency due to the chemical properties. When PEI presents in the endosome, protons are accumulated in the endosome due to the proton sponge effect of PEI. Since every third atom in PEI is a protonarable nitrogen, low pH results in osmotic swelling and rupture inside the endosomal compartment. Polymer swells by internal charge repulsion and osmotic swelling of the endosome follows. Thus, the complex can escape easily from endosome [19]. However, it has shown high cytotoxicity in different cells. Also, PEI complexes with plasmid are easy to aggregate. Various approaches have been made to reduce the cytotoxicity and increase the solubility of PEI/plasmid

complex. As modified PEI gene carrier, biodegradable PEI has been developed with difunctional PEG succinmidyol succinate [20]. Since this copolymer is degraded into small PEI and PEG due to biodegradable ester linkages in the backbone, it improves the cytotoxicity and the solubility of complexes. Insoluble lipoparticulates (ISLPs) are another example of modified PEI gene carrier. A T-shaped liponanomer was synthesized using linear PEI as a headgroup and cholesteryl chloroformate as a hydrophobic lipid anchor. ISLPs condensed plasmid efficiently and protected plasmid from degradation by nucleases [21]. Also, PEI has been conjugated with transferrin [22], galactose [23], folate [24], and NLS [25] to reduce the cytotoxic effects and aggregation.

In this study, branced PEI (25,000 Da) was used because it has been widely studied and shown efficient transfection over a wide range of cell lines.

3.2 Construction of Glucagon-Like Peptide-1 Plasmid by Using Optimal Transcriptional Regulatory Elements

3.2.1 Experimental Materials and Methods

3.2.1.1 Construction of GLP-1 Plasmids Using Different Promoters

Figure 3.2 shows the sequence of GLP-1 cDNA. A plasmid containing an expression cassette for GLP-1 (7-37) was constructed as follows. By using a DNA synthesizer, the GLP-1 (7-37) gene was synthesized. The start codon encodes methionine. If the N-terminal amino acid of the produced GLP-1 is also methionine, then GLP-1 will not be in an active form, because the first two amino acids of GLP-1 encompass its receptor binding domain. Therefore, a furin

Nhe I Furin recognition site

CTAGC**ATG**CGTCAACGTCGTCATGCTGAAGGGACCTTTACCAGTGATGTA

G**TAC**GCAGTTGCAGCAGTACGACTTCCCTGGAAATGGTCACTACAT

AGTTCTTATTTGGAAGGCCAAGCTGCCAAGGAATTCATTGCTTGGCTGGTGT

CAAGAATAAACCTTCCGGTTCGACGGTTCCTTAAGTAACGAACCGACCAC

AAAGGCCGAGGA**TAG**T

TT TCCGGCTCCT**ATC**AGATC

Xba I

Figure 3.2 Sequence of GLP-1 cDNA. Furin recognition site was underlined.

recognition site (RGRR) was introduced into the GLP-1 cDNA to produce the active form of GLP-1 (Figure 3.3). When the produced GLP-1 is secreted from the cell, the methionine is cleaved by furin in the Golgi apparatus.

Three different promoters were tested also, including the CMV promoter/enhancer, the chicken beta-actin promoter/enhancer and the SV40 promoter/enhancer.

For the CMV promoter/enhancer and the chicken beta-actin promoter/enhancer, pCI vector (Promega, Madison, WI) was used, and for the SV40 promoter/enhancer, pSI vector (Promega, Madison, WI) was used. The plasmid pβGLP-1 has chicken β-actin promoter and enhancer taken from pEBActNII. The construction process was as follows.

Schematics of plasmid containing CMV promoter/enhancer, chicken β-actin promoter/enhancer, or SV40 promoter/enhancer and GLP-1 are shown in Figure 3.4, 3.5 and 3.6, respectively.

The synthesized GLP-1 (7-37) cDNA was treated with *Nhel* and *Xbal*. In the case of the pCI-GLP1 plasmid, the protein coding sequence begins at position 1053 and terminates at position 1165.

In the case of the pSI-GLP1 plasmid, the protein coding sequence begins at position 678 and terminates at position 790. To construct this plasmid the vector was cloned into a pSI vector at *Nhel* and *Xbal* sites.

In the pβGLP1 plasmid the protein coding sequence begins at position 4016 and terminates at position 4128.

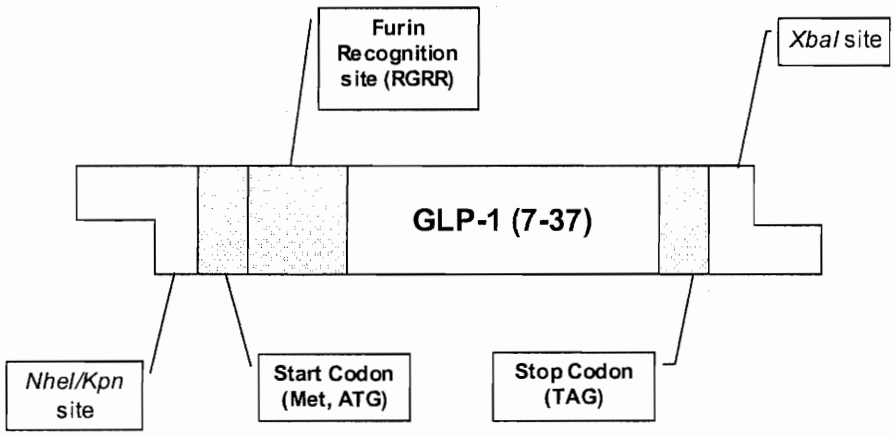

Figure 3.3 Illustration of the first step for the construction of GLP-1 plasmid.

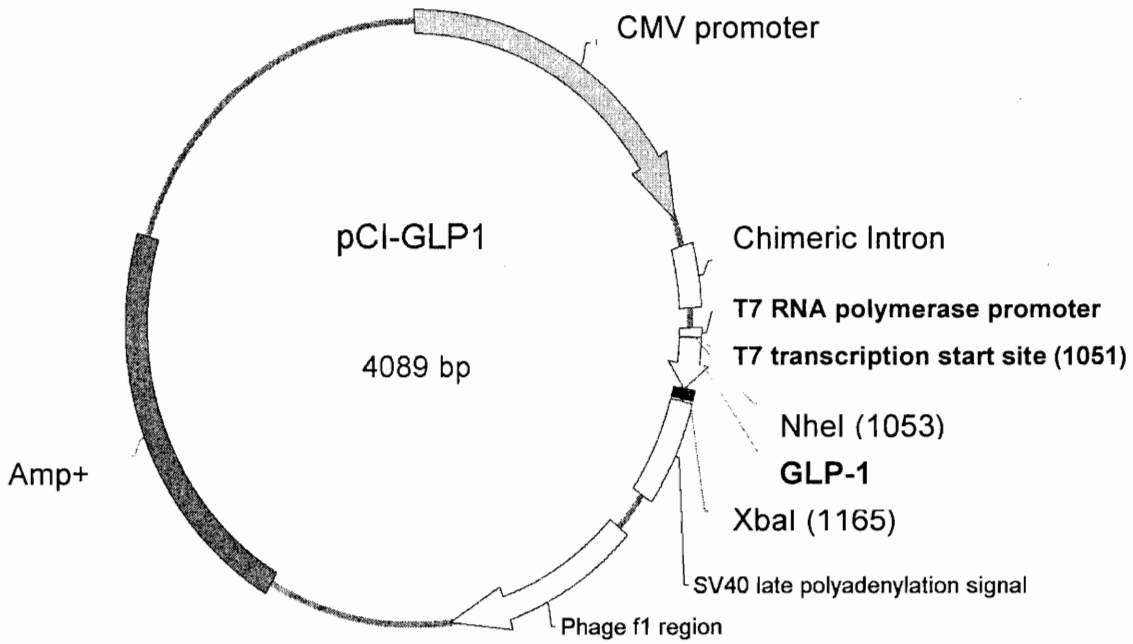

Figure 3.4 Schematic of pCI-GLP1. The structure of the pCIGLP1 plasmid. The locations of the CMV promoter and the SV40 polyadenylation sequences are shown. The furin cleavage site is between the start codon and the GLP-1 cDNA.

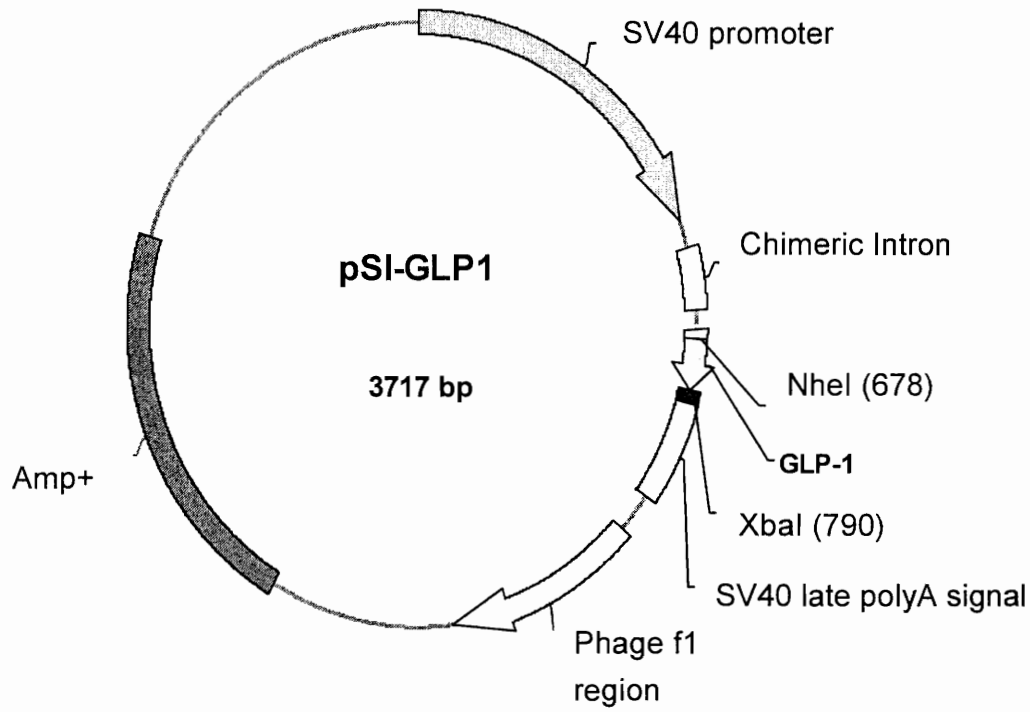

Figure 3.5 Schematic of pSI-GLP1. The structure of the pSIGLP1 plasmid. The locations of the Simian Virus 40 promoter and the SV40 polyadenylation sequences are shown. The furin cleavage site is between the start codon and the GLP-1 cDNA.

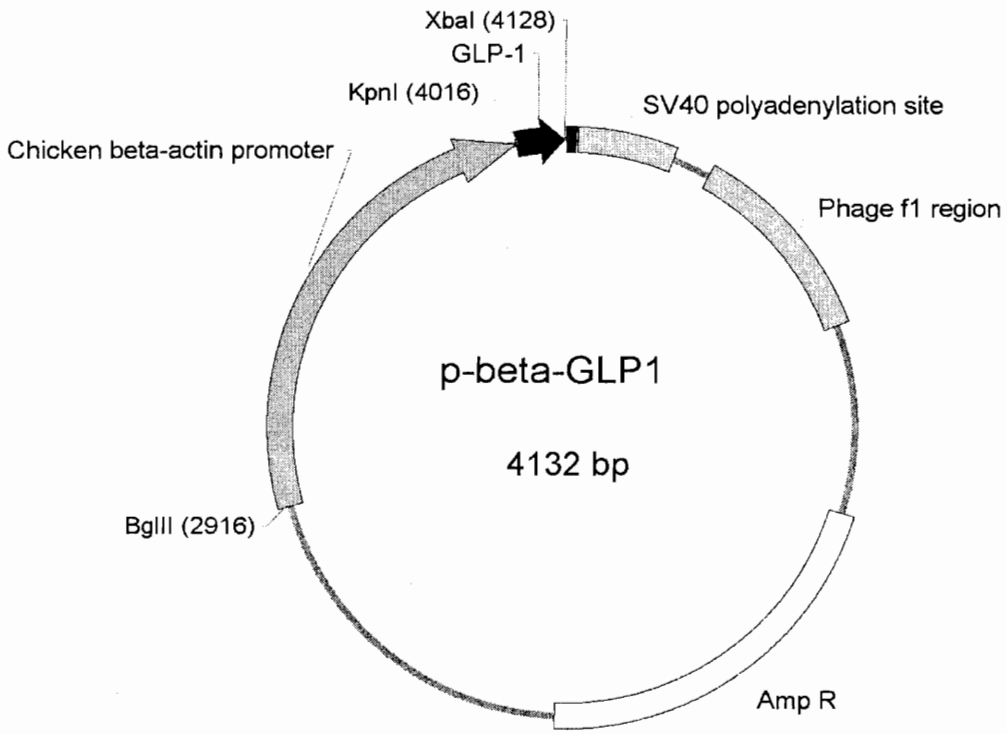

Figure 3.6 Schematic of pbeta-GLP1. The structure of the pβGLP1 plasmid. The locations of the chicken β actin promoter and the SV40 polyadenylation sequences are shown. The furin cleavage site is between the start codon and the GLP-1 cDNA.

3.2.1.2 Characterization of Plasmids

Each plasmid was confirmed by restriction mapping and partial sequencing. The pCI-GLP1 and pSI-GLP1 plasmid was digested with *NheI* and *XbaI*, and the pβ-GLP1 was digested with *KpnI* and *XbaI*. The fragments were separated using a 1% agarose gel, and then their size was identified to confirm the construction. The sequencing was performed using T7 promoter primer, which is complementary to the sequence upstream of multicloning site of each plasmid (University of Utah Health Sciences DNA sequencing facility).

3.2.1.3 Characterization of GLP-1 Plasmids with Carrier

As a carrier, polyethyleneimine (PEI) was used. The carrier/DNA complexes were prepared by self assembly. PEI (branched, 25 kDa) was purchased from Polysciences Inc. (Warrington, PA). Stock PEI solution (1.25 mg/ml) dissolved in ultra-pure water was used for all experimental procedures. The diluted carrier solution was slowly dripped into the prepared DNA plasmid solution and left for 30 minutes for formation of complex. To estimate the electric charge interaction between cationic PEI and anionic plasmid DNA, the calculated number of nitrogen atoms in PEI was divided by the phosphate atoms in DNA (N/P ratio). The PEI/DNA complex was prepared at a 5/1 N/P ratio for transfection. The formation of PEI/DNA complexes was routinely monitored by 1.0% agarose gel electrophoresis.

3.2.1.4 *In vitro* Transfection

Human hepatoma (HepG2) cell line was maintained in cell culture media supplemented with 10 % fetal bovine serum (FBS) and antibiotics (penicillin and streptomycin). The cultured cells were plated in 6 well plates at initial density of 5 $\times$ 10^5 cells/well for 24 hours prior to transfection. All experiments were performed in triplicate. The transfection was carried out in serum-free media. After 24 hours, the cells were washed twice with serum-free medium, and then 2 ml of fresh serum-free medium was added. The polymer/plasmid complexes were added to the cells. The cells were then incubated for 4 hours at 37 °C in 5 % CO_2 and 95 % air incubator. The amount of plasmids for transfection was 6 µg per each well. After 4 hours, the transfection mixtures were removed and 2 ml of fresh media with 10 % FBS was added. The cells were incubated for an additional 48 hours, and samples were prepared for the GLP-1 assay.

3.2.1.5 ELISA of GLP-1

To evaluate the amount of GLP-1 released, the concentration of the GLP-1 in the medium was determined by ELISA using GLP-1 (active) ELISA kit (Linco, St. Charles, MO). The expressed GLP-1 should be secreted in its active form for therapeutic purposes. To assess this, an ELISA assay for the active form of GLP-1 was performed 48 hours after transfection. This assay has been developed to measure biologically active glucagon-like peptide-1 (7-36, 7-37) in plasma, serum or tissue culture medium. It is a noncompetitive immunoassay based on enzyme labeled quantification of GLP-1 detected by a fluorogenic substrate. Since the

amount of fluorescence generated is directly proportional to the concentration of active GLP-1 in the unknown sample, the latter can be derived by interpolation from a reference curve generated in the same assay with reference standards of known concentrations of active GLP-1. The plate was read by a fluorescence plate reader with an excitation / emission wavelength of 355nm / 460nm.

3.2.1.6 Statistical Analysis of ELISA Data

The amount of GLP-1 produced from different amounts of each plasmid was compared. Data from ELISA were analyzed by two-way ANOVA for repeated measures using a generalized linear model. In all cases a p value of <0.05 was considered to be statistically significant.

3.2.2 Results

3.2.2.1 Construction of GLP-1 Plasmid

In this study, it was hypothesized that a GLP-1 gene delivery system could sustain the protein release in the body to overcome the major drawback of short half-life of GLP-1. However, creating a system that would produce the active form of GLP-1 requires an extra step. The first two amino acids of GLP-1 (7-37) are important because it is a receptor binding domain. If the start codon, which encodes methionine, is placed just before GLP-1 cDNA in the plasmid, the active form of GLP-1 (7-37) cannot be produced because of a masked receptor binding domain. As mentioned in the introduction, a GLP-1 minigene containing

plasmid was tested for *in vitro* transfection of mouse insulinoma cells [1-2]. They used a natural posttranslational process to make active GLP-1 in pancreatic islet β-cells. In this study, a furin cleavage site was placed between the start codon and GLP-1 cDNA to produce the active form of GLP-1. Furin is one of the endoproteases found in the Golgi apparatus in most mammalian cells.

3.2.2.2 Production of GLP-1 in Transfected HepG2 Cells

The expressed GLP-1 should be secreted in its active form for therapeutic purposes. To assess this, an ELISA assay for the active form of GLP-1 was performed 48 hours after transfection. The active form of GLP-1 was detected in the cell culture media by ELISA assay, suggesting that the expressed GLP-1 was secreted into the cell culture media by GLP1 plasmids. 6 μg of pSIGLP1 transfected HepG2 cells were assayed for GLP-1 production produced 4 fold more GLP-1 than that from 6 μg pCIGLP1 transfected HepG2 cells (27.5 ± 4.5 ng/L vs. 129.3 ± 16.2 ng/L) (Figure 3.7).

3.2.3 Discussion

Generally the level of expression of a foreign gene from a recombinant plasmid is influenced by the number of genes in the cell, the rate of transcription of the gene, and the stability of the mRNA transcription. It has been known that the gene expression level is mainly regulated by the level of transcription. Therefore, the optimization of transcriptional regulatory elements is required to obtain higher gene expression. The elements involved in transcription process

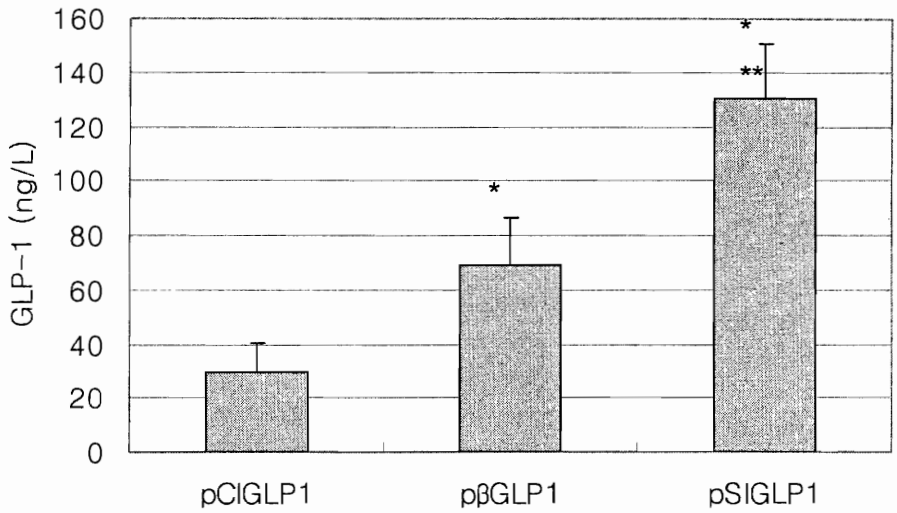

Figure 3.7 *In vitro* transfection assay in HepG2 cells for GLP-1 expression. The GLP-1 levels after transfection of the PEI/GLP1 plasmid complex into HepG2 cells (6×10^5 cells per well), the media was collected and the active GLP-1 level was measured by ELISA. The graph represents the average ± SE of six experiments. *P<0.05 as compared to pCIGLP1, **P<0.05 as compared to pβGLP1.

include the enhancer and promoter. Enhancers are cis-acting DNA segments stimulating transcription from promoters [26]. The presence of an active enhancer results in an increased density of RNA polymerase II molecules at the linked gene leading to a higher transcription rate [27].

In this study, to compare relative promoter/enhancer strengths, three plasmids containing different promoters/enhancers were constructed and tested *in vitro*. The strength of each promoter/enhancer has been tested indirectly by monitoring the GLP-1 gene activity following direct introduction into the cell via transfection of the plasmid/carrier complex into the cells. The comparison of different promoters/enhancers showed that the SV40 promoter/enhancer yielded the highest expression in cultured HepG2 cells.

The SV40 enhancer is represented by a DNA sequence of 72 bp with multiple motifs connected with transcriptional activation [28]. Some proteins recognizing these motifs have been identified as transcriptional activators [29]. In other words, these sequences have affinity for nuclear transcription factors.

3.3 Modification of Glucagon-Like Peptide-1 Plasmid with Nuclear Factor Kappa-B Binding Sites

3.3.1 Experimental Materials and Methods

3.3.1.1 Construction of Plasmid with Nuclear Factor Kappa-B Binding Sites

To modify previously constructed plasmids, NFκB binding sites were inserted after the SV40 polyadenylation site of each plasmid (Figure 3.8). A

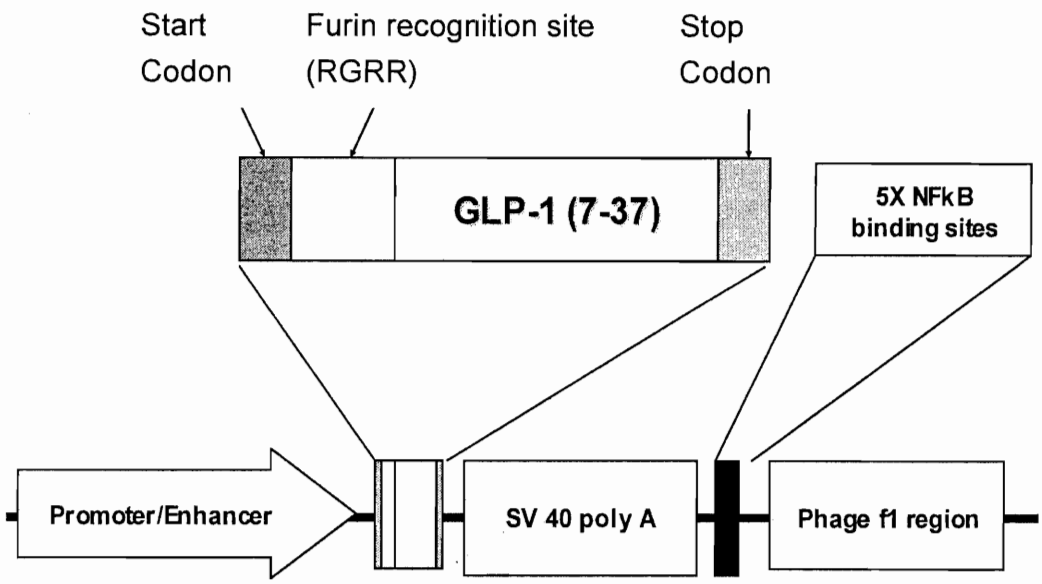

Figure 3.8 Illustration of GLP-1 plasmid modification with NFκB binding sites

fragment consisting of five direct repeats of the IκB motif, 5'-GGGGACTTCC-3' was synthesized using a DNA synthesizer.

Each plasmid was confirmed by restriction mapping and partial sequencing. The pCI-GLP1-NFκB and pSI-GLP1-NFκB plasmid was digested with Bgl II and XbaI, and the pβ-GLP1-NFκB was digested with KpnI and XbaI. The fragments were separated using a 1% agarose gel, and then their size was identified to confirm the construction. The sequencing was performed using T7 promoter primer, which is compliment to the sequence upstream of multicloning site of each plasmid.

3.3.1.2 *In vitro* Transfection

Human hepatoma (HepG2) cells were maintained in the cell culture media with 10 % FBS and antibiotics (penicillin and streptomycin). Cells were grown in same environment. Transfection process and sample preparation was same as the previous *in vitro* transfection.

After washing transfected cells with PBS, the total RNA was prepared by acid-guanidium thiocyanate-phenol- chloroform extraction, using RNAwiz (Ambion, Austin, TX). Any residual DNA was removed from RNA by incubating the RNA extract with DNase (Promega, Madion, WI) at 37°C for 30 minutes. The concentration of RNA was measured by the absorbance at 260 nm and 280 nm. Two microgram of the total RNA was hybridized to the backward primer and reverse transcribed using AMV reverse transcriptase (Promega, Madion, WI). The reverse transcribed samples will be amplified by polymerase chain reaction (PCR), using Taq polymerase (Promega, Madison, WI). The sequences of the

specific oligonucleotide primers are as follows: GLP-1 forward primer, 5'-CAGAAGTTGGTCGTGAGGCA-3'; GLP-1 backward primer, 5'-GCCTTTCACCAGCCAAGCAA-3'. The PCR reaction consists of 94 °C for 5 minutes, 25 cycles at 94 °C for 30 seconds, 55 °C for 30 seconds, 72 °C for 1 minute, followed by an extension of 10 minutes at 72 °C. The size of the expected product is 112 bp for GLP-1.

3.3.1.5 *In vitro* Biological Activity Assay of GLP-1

To determine if insulin secretory action of GLP-1 is glucose dependent, islet cells were cultured with the GLP-1 transfected cells under various glucose concentrations.

3.3.1.5.1 Isolation of Rat Islets

Islets of Langerhans were isolated from male SD rat pancreas by a collagenase digestion technique. Each rat was fasted for approximately 12 hours prior to the islet isolation procedure with water available *ad libitum*. After anesthetizing rats, 5-10 ml of blood was removed by a cardiac puncture to avoid blood contamination during the dissection procedure. The pancreas was exposed by using a midline incision. A collagenase solution in Hank's Balanced Salt Solution (HBSS) 4-8 ml was injected into the pancreas via the common bile duct.

The pancreas was dissected and incubated at 37 °C for 5-10 minutes with gentle vortexing every 2 minutes. The digested tissue was rinsed twice with cold HBSS at 4 °C in order to stop the tissue digestion process. The pancreatic

tissue was passed through a 500-μm mesh. The islets were purified by using a Ficoll density gradient. The islets were rinsed twice with HBSS to remove the toxic Ficoll solution. The islets were cultured in a medium of RPMI-1640 complete 100G medium. RPMI-1640 complete 100G medium contains RPMI-1640 supplemented with 100-mg/dl glucose, antibiotics (penicillin, streptomycin, amphotericin B), and 10% FBS. The islets were incubated in the medium for one day at 37 °C in a 95% air 5% CO_2 incubator. The islets were handpicked under using a light microscope at 50X magnification. The handpicked islets were again incubated under the same conditions for 24 to 48 hours prior to their use in co-culture experiment.

3.3.1.5.2 Co-culture of Isolated Rat Islets with the Transfected Cells

The cells were cultured and transfected with each plasmid by using the same procedure as described previously. The 30 islets were carefully transferred into the transfected cell culture system by using cell culture insert for a physical separation. Then, the culture medium was changed to fresh RPMI-1640 medium with different glucose content (50 mg/dl and 300 mg/dl). After 4 hours of co-incubation, the culture medium was collected for GLP-1 and insulin assay. This experiment was repeated until the result can account for statistical significance (Statistical analysis is in the next section).

3.3.1.5.3 RIA of Insulin

The radioimmuno assay is based upon the competition of 125-I-peptide and peptide (either standard or unknown) binding to the limited quantity of antibodies specific for the peptide in each reaction mixture. As the quantity of standard or unknown in the reaction increases, the amount of 125-I-peptide able to bind to the antibody is decreased. By measuring the amount of 125-I-peptide bound as a function of the concentration of peptide in standard reaction mixtures, it is possible to construct a standard curve from which the concentration of peptide in unknown samples can be determined. Thus, four basic components are necessary for a radioimmunoassay system which include following. (1) a specific antiserum to the antigen to be measured (2) a radioactive labeled form of the antigen (3) a method whereby antibody-bound tracer can be separated from the unbound tracer (4) an instrument to count radioactivity, which is the gamma counter in the CCCD laboratory, University of Utah. In this study, the plasma insulin level was determined by using Insulin RIA kit (ICN Pharmaceuticals, Costa Mesa, CA).

3.3.1.5.4 Statistical Analysis

The amount of insulin produced from different glucose concentration was compared. Two-way ANOVA for repeated measures (Generalized Linear Model) was used to analyze data from Insulin RIA. In all cases a p value of <0.05 was considered to be statistically significant.

3.3.2 Results

3.3.2.1 Production of GLP-1 in Transfected HepG2 Cells

The expressed GLP-1 should be secreted in its active form for therapeutic purposes. To assess this, an ELISA assay for the active form of GLP-1 was performed 48 hours after transfection. Figure 3.9 shows the amount of GLP-1 in each medium after transfection with each plasmid. Two micrograms of plasmid DNA complexed with PEI at the N/P ratio 5:1 were used to transfect the cells in 6-well culture plates. Forty eight hours after transfection, the concentrations of GLP-1 in culture media measured by ELISA showed approximately a 5 fold higher level in the pSIGLP1NFκB transfected cells than in the pCIGLP1 transfected cells.

3.3.2.2. Expression of GLP-1 mRNA in Transfected HepG2 Cells

The pSIGLP1/PEI and pSIGLP1NFκB/PEI complex was transfected into HepG2 cells to evaluate its expression of GLP-1. The sequences of the specific oligonucleotide primers were as follows: GLP-1 forward primer, 5'-CAGAAGTTGGTCGTGAGGCA-3'; GLP-1 backward primer, 5'-GCCTTTCACCAGCCAAGCAA-3'. As a result, the GLP-1 mRNA was detected in the pSIGLP1 and pSIGLP1NFκB plasmid transfected cells (Figure 3.10).

This result suggests that the detected mRNA was expressed by the exogenous transferred pSIGLP1 and pSIGLP1NFκB plasmid, not by the endogenous GLP-1 gene.

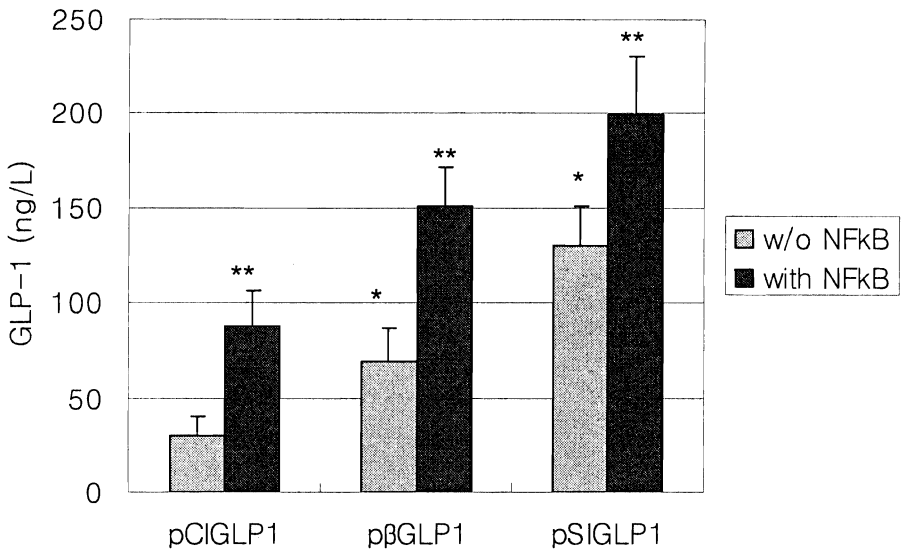

Figure 3.9 *In vitro* transfection assay in HepG2 cells for GLP-1 expression. The GLP-1 levels after transfection of the PEI/GLP1 plasmid complex into HepG2 cells (6×10^5 cells per well), the media was collected and the active GLP-1 level was measured by ELISA. The graph represents the average ± SE of six experiments. *P<0.05 as compared to pCIGLP1, **P<0.05 as compared to GLP-1 plasmid without NFκB binding sites.

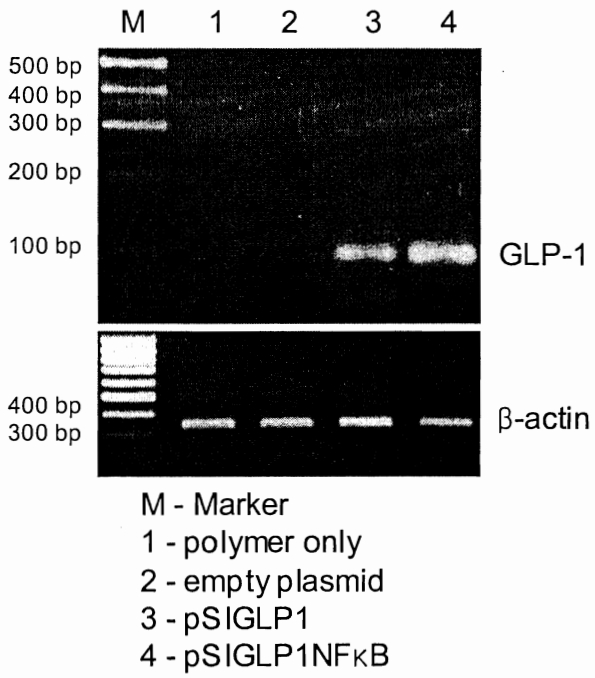

Figure 3.10 Reverse transcriptase-polymerase chain reaction (RT-PCR). M, molecular marker; Lane 1, polymer only; Lane 2, empty plasmid; Lane 3, PEI/pSIGLP1; Lane 4, PEI/pSIGLP1NFκB.

3.3.2.3 *In Vitro* Biological Activity Assay of GLP-1

It was studied whether the secreted GLP-1 from the transfected HepG2 cells can stimulate secretion of insulin from isolated rat islets. The isolated rat islets were cultured with the pSIGLP-1 and pSIGLP1NFκB transfected HepG2 cells. There was no enhancement of insulin secretion under low glucose concentration (50 mg/dL). However, a remarkable increment of insulin secretion occurred under high glucose concentration (300 mg/dL) (Figure 3.11). The transfected HepG2 cells in each well produced 13.2 μg/L GLP-1 and 16.3 μg/L GLP-1 over 4 hours, respectively. These results showed that GLP-1 significantly stimulated the secretion of insulin under high glucose conditions but not under low glucose conditions.

3.3.3 Discussion

Non-viral delivery of GLP-1 plasmid was proposed for treatment of diabetes. The goal of this study was to increase GLP-1 expression level by modifying GLP-1 plasmid with NFκB binding sites.

There have been several attempts to improve nuclear transport of plasmid DNA, such as electrostatic binding of DNA to NLS-containing proteins [30, 31] or peptides [32] or lipids [33], as well as sequence-specific binding of DNA to karyophilic proteins [34]. However, the stability of those complexes has been unpredictable. NLS sequences are positively charged and interact strongly with DNA backbones. NFκB is endogenous NLS-protein which can bind to specific

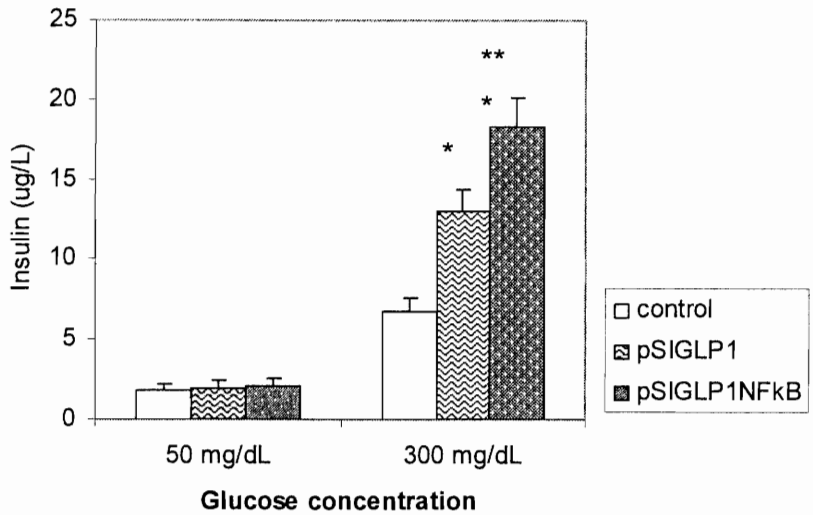

Figure 3.11 Insulin production in co-culture isolated rat islets with pSIGLP1 transfected HepG2 cells. Two days after gene transfection, the co-culture (30 isolated rat islets with 6×10^5 HepG2 cells) was performed under two different conditions; 2.7 and 16.5 mmol/L of glucose concentration in media. The graph represents the average ± SE of six experiments. *P<0.05 as compared to control under high glucose condition, **P<0.05 as compared to pSIGLP1 under high glucose condition.

DNA sequence elements. Binding affinity of NFκB to its DNA binding sites is high, and complex formation between NFκB and the plasmid DNA may protect the DNA from entering into endocytic pathway.

The ability of NFκB to mediate delivery of biologically active GLP-1 plasmid DNA has been tested indirectly by monitoring the GLP-1 gene activity following direct introduction into the cell via transfection of the plasmid with NFκB binding sites into the cells.

The plasmid of pSIGLP1NFκB showed higher gene activity than pSIGLP1 with an increase of 1.5-fold. The enhancement by using NFκB binding sites was relatively smaller than cases with pCIGLP1 and pβGLP1. This result could be explained by the fact that the SV40 enhancer can mediate nuclear transfer of the DNA by binding to ubiquitously expressed transcription factors.

By RT-PCR, it was shown that the detected mRNA was expressed by the exogenously transferred pSIGLP1 and pSIGLP1NFκB plasmid, not by the endogenous GLP-1 gene.

Co-culture assay of the GLP-1 plasmid transfected cells with isolated rat islet cells demonstrated that GLP-1 increased insulin secretion from transfected HepG2 cells, compared to controls during a hyperglycemic challenge. Produced GLP-1 has an insulinotropic effect under high glucose concentrations (300 mg/dL) but not under low glucose concentrations (50 mg/dL). These results are clinically desirable and support the safety of the GLP-1 treatment because there is no risk of hypoglycemia.

3.4 Conclusions

To achieve therapeutic activity of GLP-1, the expression level should be high enough to produce sufficient level of GLP-1 for lowering high glucose level of diabetic subjects. The aim of this study was to design a new and effective GLP-1 delivery system for therapeutic use for type 2 diabetes treatment based upon construction of efficient GLP-1 plasmid. For effective gene delivery, expression efficiency should be enhanced. The expression levels of the introduced gene depend mainly on the strength of transcriptional regulatory elements and the transduction efficiency of the gene transfer vector. In this study, the main focus was on the strength of promoter/enhancer and enhancement of nuclear import.

First, three plasmids containing different promoters/enhancers were constructed and tested *in vitro* to compare relative promoter/enhancer strengths. The strength of each promoter/enhancer was tested indirectly by monitoring the GLP-1 gene activity following direct introduction into the cell via transfection of the plasmid/carrier complex into the cells. The comparison of different promoters/enhancers showed that the SV40 promoter/enhancer yielded the highest expression in cultured HepG2 cells.

Then, GLP-1 plasmid was modified with NFκB binding sites. The ability of NFκB to enhance transport of GLP-1 plasmid DNA has been tested by monitoring the GLP-1 gene activity following transfection of the plasmid into the cells. After transfection, the concentrations of GLP-1 showed approximately a 5

fold higher level in the pSIGLP1NFκB transfected cells than in the pCIGLP1 transfected cells.

As shown by the data, more active GLP-1 was expressed after transfection of newly designed GLP-1 plasmid with SV40 promoter/enhancer than plasmid with CMV promoter/enhancer or β-actin promoter/enhancer. Also, modification of GLP-1 plasmid with NFκB binding sites results in increase of expression level. Biological activity of produced GLP-1 after transfection was tested and it was shown that GLP-1 has an insulinotropic effect under high glucose concentration but not under low glucose concentration. These results suggest the safety of the GLP-1 treatment without any risk of hypoglycemia. Therefore, the selected plasmid will be tested with type 2 diabetic animal model to study the feasibility of GLP-1 gene delivery as treatment of type 2 diabetes.

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CHAPTER 4

GLUCAGON-LIKE PEPTIDE-1 GENE DELIVERY IN TYPE 2 DIABETIC ANIMAL MODEL

4.1 Introduction

Glucagon like peptide-1 (GLP-1) is a potent insulinotrophic hormone, which makes GLP-1 an attractive candidate for the treatment of type 2 diabetes. However, the short plasma half-life of the active forms of GLP-1 poses an obstacle to the sustained delivery of this peptide. In this study, the effect of GLP-1 gene delivery *in vivo* using a new plasmid constructed with a GLP-1 (7-37) cDNA was studied. This cDNA contains a furin cleavage site between the start codon and the GLP-1 coding region. The expression of the GLP-1 gene in this study is driven by a SV-40 promoter. For type 2 diabetic animal model, diet induced obese (DIO) mice were used.

Type 2 diabetes is characterized by excessive hepatic glucose production, decreased insulin secretion, and insulin resistance [1]. Elevated glucose level causes increased oxidative stress due to increased production of reactive oxygen species (ROS) [2]. The oxidative stress leads to the activation of stress-sensitive signaling pathways and worsens insulin secretion and action. NFκB is a major

intracellular target of hyperglycemia and oxidative stress. Activators of NFκB include elevated glucose, ROS, tumor necrosis factor-α (TNF-α), and other proinflammatory cytokines [3].

One of the molecules that accumulates in diabetic patients is TNF-α. It has been shown that more amount of TNF-α produced in type 2 diabetic patients. A dramatic increase in the TNF-α release has been reported to be mediated by ROS via activation of transcription factor NFκB in cultured cells incubated with high glucose for 18 hours [4]. The production of TNF-α depresses the synthesis of glut 4, and the loss of glut 4 inhibits an insulin stimulated cell from glucose uptake. TNF-α is the major cytokine, which stimulates NFκB effectively. Therefore, new GLP-1 plasmid was designed by incorporating NFκB binding sites to GLP-1 plasmid for the treatment of diabetes. Figure 4.1 shows the schematic of NFκB activation and its subsequent nuclear entry. In diabetic subjects, high glucose level and TNF-α phosphorylate IκB. Thus, NFκB is activated by phosphorylation and degradation of IκB.

4.2 Experimental Animals

Male C57BL/6J mice, 4 weeks of age, were used to study the effect of GLP-1 plasmid on type 2 diabetic subjects. Mice were purchased from Jackson Laboratory (Bar Harbor, ME). To make a fully developed insulin resistant diet induced obese (DIO) animal phenotype, 50 animals were fed with a high fat and high calorie diet (60 kcal% fat, D12492, Research Diet, New Brunswick, NJ) for 3 months. After 3 months, these animals showed the phenotype of metabolic

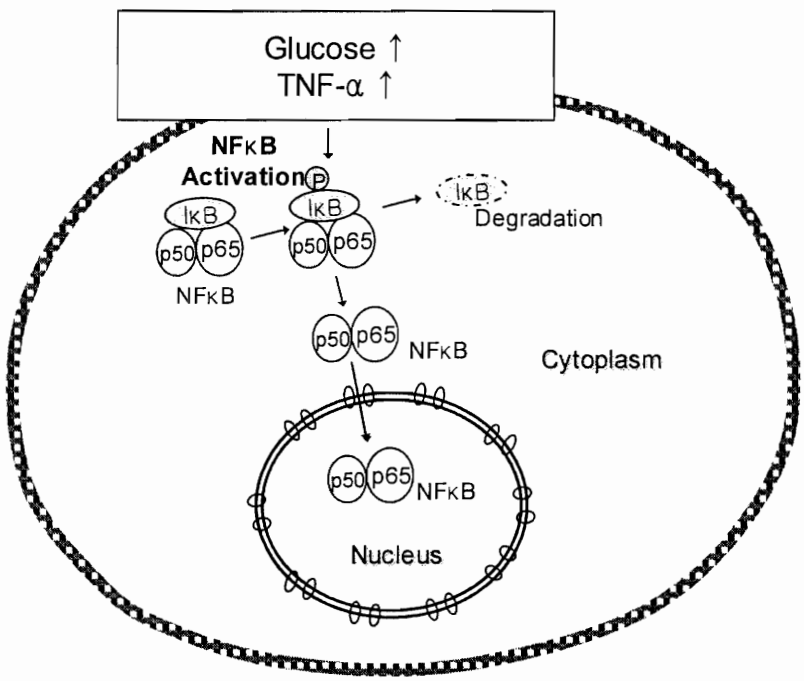

Figure 4.1 Model of NFκB activation in diabetes mellitus.

syndrome characterized by obesity, high blood glucose and low plasma insulin level. Blood samples were obtained from the tail vein for glucose, GLP-1 and insulin assays. Animals were maintained under virus and antibody free conditions in climatized rooms with free access to sterilized tapwater and special food for DIO mice.

4.3 Experimental Materials and Methods

4.3.1 Injection of GLP-1 Plasmid with PEI

All animals were kept under specific pathogen free conditions in the animal facility. Anesthesia was induced by intramuscular injections of pentobarbital (6 mg/kg). The experimental animals, Male DIO mice, 4 months old, were divided into five groups. Each group consisted of fifteen mice for three sets of assay. In addition to blood sampling, IPGTT was done 2 days after injection. The first group was not injected. The second group was injected with the carrier PEI only. The third group was injected with the PEI/empty plasmid complex, with the N/P ratio of 5/1. The next two groups were injected with the PEI/pSIGLP1 and PEI/pSIGLP1NfκB plasmid complex. Blood samples were withdrawn at day 0, 2, 4, 7, 10, 14, 17 and 21 after injection to measure blood glucose, GLP-1, and insulin concentrations.

4.3.2 Assessment of Glucose Level

Blood glucose level was tested using glucometer (Accu-check Advantage from Roche Diagonistics Corporation, Indianapolis, IN). 10 μl of blood is required for each measuring.

4.3.3 Assessment of GLP-1 Level

To evaluate the efficacy of GLP-1 gene delivery, the concentration of the GLP-1 in the plasma was determined by ELISA using GLP-1 (active) ELISA kit (Linco, St. Charles, MO).

4.3.4 Assessment of Insulin Level

To evaluate the efficacy of GLP-1 gene delivery, the concentration of insulin in the plasma was determined by RIA using Insulin RIA kit (ICN Pharmaceuticals, Costa Mesa, CA).

4.3.5 Statistical Analysis

Data for the effect of GLP-1 gene therapy in type 2 diabetic animal model was analyzed by 2-way ANOVA for repeated measures using a generalized linear model. In all cases a p value of <0.05 was considered to be statistically significant.

4.3.6 Intraperitoneal Glucose Tolerance Test

A glucose tolerance test was used to evaluate the ability of the animals to tolerate a standard glucose load. Mice (n=5 each at 2 days after injection) were fasted overnight before the intraperitoneal glucose tolerance test. A pre-load blood sample will be taken and a glucose load of 1g/kg was administered via intraperitoneal injection. Subsequent blood samples were taken 0, 15, 30, 60, and 120 minutes after injection. Blood samples were obtained from the tail vein

and used for measuring the levels of insulin. This procedure allowed for collection without catheterization of blood vessel. All animals were anesthetized by intramuscular injections of pentobarbital (6 mg/kg) before the glucose injection.

4.4 Results and Discussion

The pSIGLP1/PEI and pSIGLP1NFκB/PEI (N/P ratio 5:1) complex (200 µl) was injected into the mice via tail veins. All mice tolerated the injections well, and no injection-related deaths occurred. It was studied whether the delivered pSIGLP1NFκB plasmid can decrease blood glucose levels in DIO mice, and the effect of incorporating NFκB binding sites into plasmid. After intravenous administration of 200 µg of the PEI/pSIGLP1NFκB complex, the blood glucose levels began to decrease after the injection (Figure 4.2). This decrease continued until the second day following administration, after which the blood glucose levels increased until the 21th day after injection. However, the blood glucose levels did not return to the preadministration base line until the 17th day after injection. The control groups showed no significant change in their blood glucose levels during a similar time period. The second group of animals that received pSIGLP1/PEI showed a similar pattern of glucose level change as the pSIGLP1NFκB/PEI group, but the change of blood glucose in the pSIGLP1 group was relatively smaller than pSIGLP1NFκB group.

The plasma level of GLP-1 from each group was also monitored. In conjunction with the changes in blood glucose levels, the PEI/pSIGLP1NFκB group began to increase plasma GLP-1 concentration at the second day after

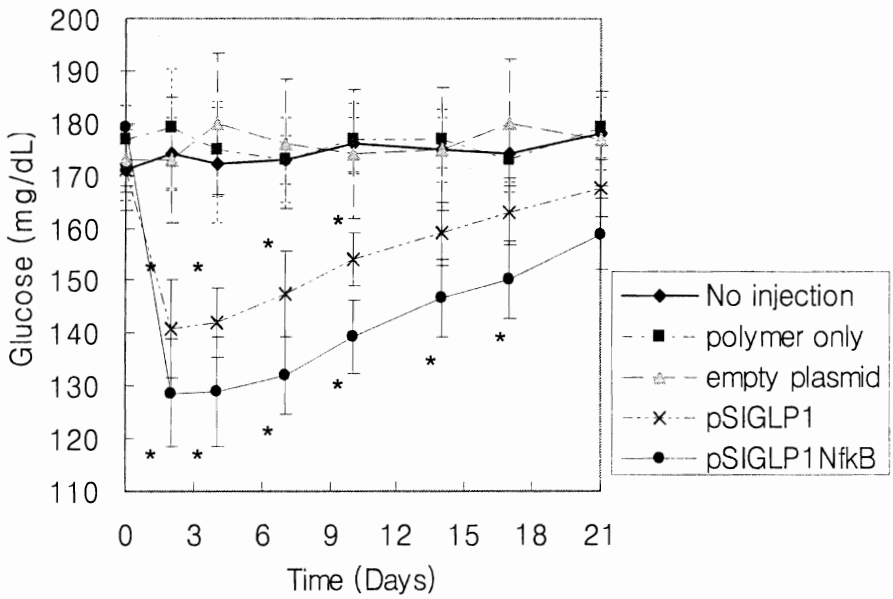

Figure 4.2 Blood glucose level changes after injection of PEI/GLP-1 plasmid. The DIO mice received intravenous injection of PEI (square) or PEI/empty plasmid (triangle) or PEI/pSIGLP1 (x), or PEI/pSIGLP1NFκB (circle). Each group was composed of six mice, and the graph represents the average ± SE. *P<0.05 as compared to control.

injection (Figure 4.3). The GLP-1 level steadily decreased through the 28th day of the study. The control groups showed almost no changes in GLP-1 concentration.

The plasma insulin level was assayed to see the insulinotropic effect of GLP-1. The pattern of the insulin concentration change in the PEI/pSIGLP1NFκB group mirrored the temporal profile of the plasma GLP-1 levels. This group's plasma insulin concentration value increased 2.5 fold above the baseline (Figure 4.4). The control groups showed minimal changes in plasma insulin concentration.

Two days after the complex was injected, plasma GLP-1 values increased dramatically, and these values gradually returned to baseline levels after 3 weeks (Figure 4.3). Blood glucose levels also decreased, and returned to preinjection levels after 3 weeks (Figure 4.2). Plasma insulin level also increased, and gradually returned to baseline 17 days after injection (Figure 4.3). An intraperitoneal glucose tolerance test (IPGTT) was performed to verify the improvement of glucose tolerance. The blood glucose level showed marked decrease (Figure 4.5).

The amount of food consumption and the change in body weight after injection was also monitored. Food consumption decreased 2 days after injection, and gradually returned to preinjection values after 14 days (Figure 4.6). Body weight also decreased after injection (Figure 4.7). The time sequences of the changes of parameters showed close correlation with the changes of the plasma GLP-1 levels. After continuous subcutaneous infusion of GLP-1, patients with

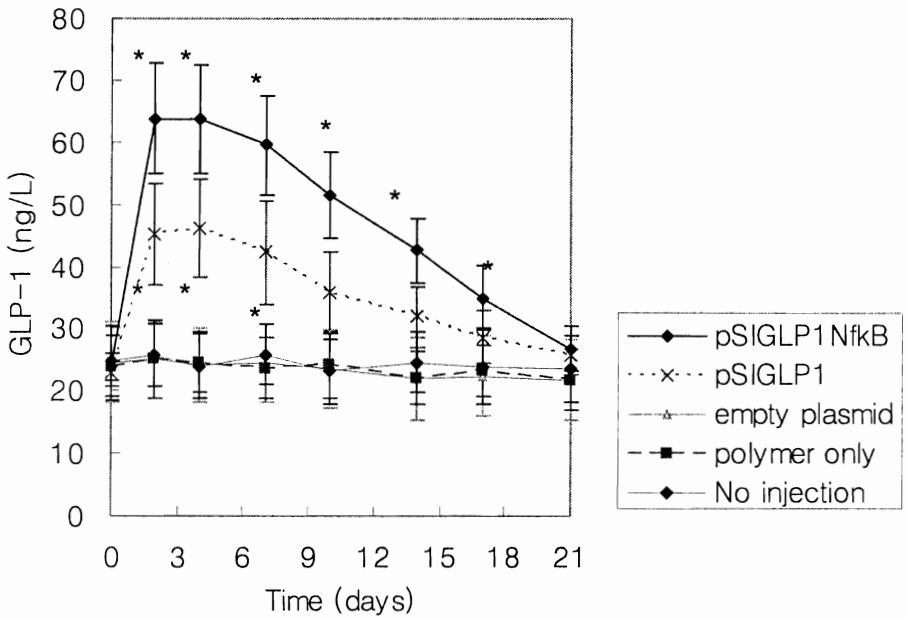

Figure 4.3 Plasma GLP-1 level changes after injection of PEI/GLP-1 plasmid. The DIO mice received intravenous injection of PEI (square) or PEI/empty plasmid (triangle) or PEI/pSIGLP1 (x), or PEI/pSIGLP1NFκB (circle). Each group was composed of six mice, and the graph represents the average ± SE. *P<0.05 as compared to control.

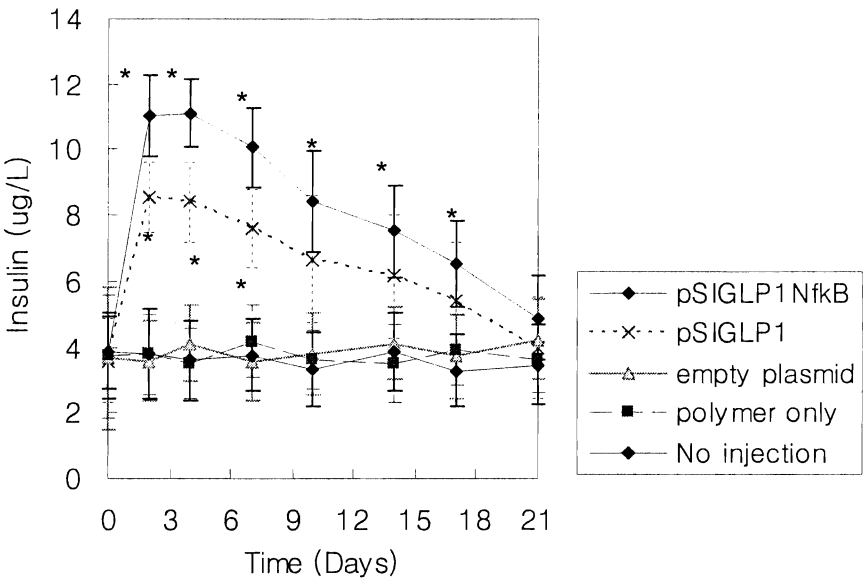

Figure 4.4 Plasma insulin level changes after injection of PEI/GLP-1 plasmid. The DIO mice received intravenous injection of PEI (square) or PEI/empty plasmid (triangle) or PEI/pSIGLP1 (x), or PEI/pSIGLP1NFκB (circle). Each group was composed of six mice, and the graph represents the average ± SE. *P<0.05 as compared to control.

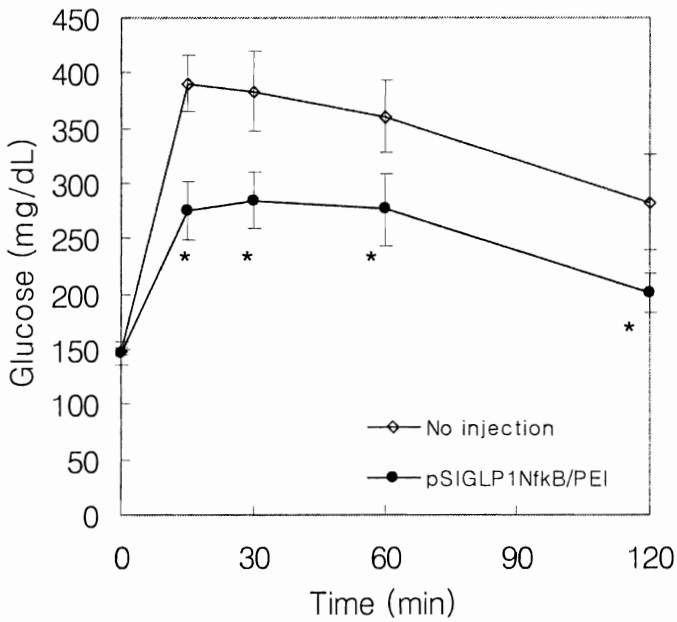

Figure 4.5 Blood glucose level in DIO mice after glucose challenge (IPGTT). At day 2, blood glucose levels in mice treated with nothing (open circle) and PEI/pSIGLP1NFκB (closed circle) were assayed for the indicated times after intraperitoneal glucose injection. The graph represents the average ± SE, each group was composed of six mice. Statistical significance between groups (control vs. PEI/pSIGLP1NFκB) was determined by repeated-measures ANOVA: *P<0.05 as compared to control.

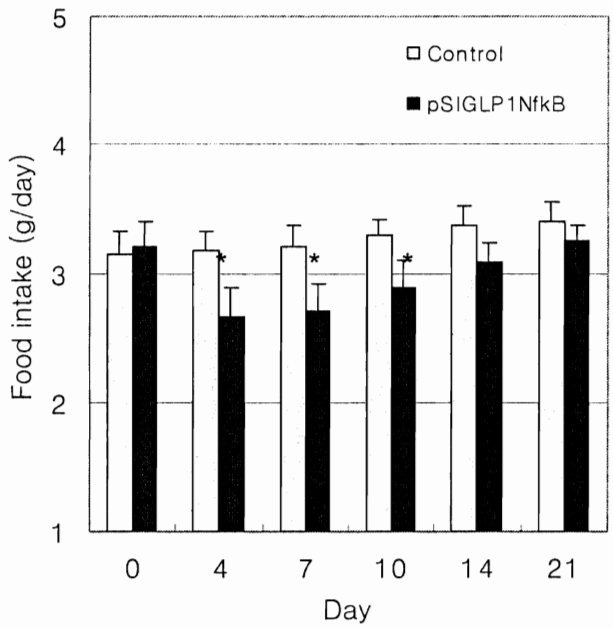

Figure 4.6 Change in food consumption was monitored. Each group was composed of six mice, and the graph represents the average ± SE. *P<0.05 as compared to control.

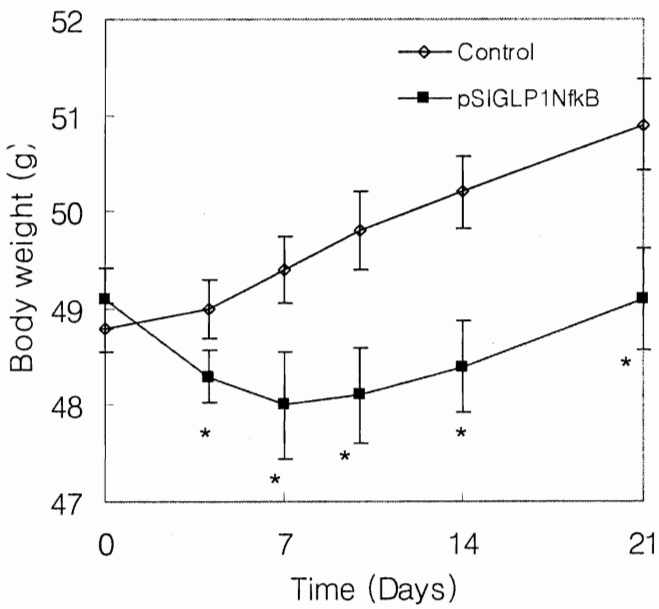

Figure 4.7 Change in body weight was monitored. Each group was composed of six mice, and the graph represents the average ± SE. *P<0.05 as compared to control.

type 2 diabetes reported a reduction in appetite, which led to significant reductions in body weight [5]. Several reasons seem to be involved with reductions in body weight. First, GLP-1 decreases gastric emptying rates. However, the reduced sensation of appetite was reported not only in the postprandial state, but also in the fasting state and before meal ingestion in humans [6]. This suggests mechanisms other than decreased gastric emptying also contribute to body weight reduction. It has been shown that the central administration of GLP-1 inhibits food intake in rodents [7]. Since circulating GLP-1 can access GLP-1 receptors in brain, it will participate in the regulation of appetite [8]. It is also possible that gastric distension activates GLP-1 containing neurons, so it can act as an inhibitor of food intake [9].

4.5 Conclusions

GLP-1 is very promising candidate for type 2 diabetes treatment. GLP-1 has many advantages for treating type 2 diabetes. However, there is a major limitation to clinical applications due to the extremely short half-life of GLP-1 *in vivo*. It has been suggested that continuous infusion of GLP-1 can normalize hyperglycemia in type 2 diabetes [10]. In this study, GLP-1 plasmids with NFκB binding sites was delivered and showed GLP-1 expression *in vivo* for an extended time. A single injection of GLP-1 plasmid/PEI complex into DIO mice resulted in increasing insulin secretion and decreasing blood glucose level that was maintained for more than 2 weeks. Body weight was also decreased after injection of GLP-1 plasmid/PEI complex. Since there is no risk of hypoglycemia

under higher concentrations of GLP-1, a potent promoter/enhancer, SV40 promoter/enhancer, was used for GLP-1 plasmid. GLP-1 is produced in intestinal L-cells in the body. GLP-1 plasmid was delivered systemically by intravenous injection. A furin cleavage site was inserted in the GLP-1 coding region in order to separate methionine which the start codon encodes. Thus, active form of GLP-1 could be produced after delivery of plasmid/polymer complex.

In conclusion, GLP-1 plasmid was designed for high expression level and as a result of its delivery blood glucose level was significantly lowered in a type 2 diabetic animal model. Also, delivered GLP-1 gene showed insulinotropic activity. Therefore, it is proposed that design of GLP-1 plasmid and its delivery can be effectively used for treatment of type 2 diabetes.

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CHAPTER 5

SUMMARY AND FURTHER RESEARCH

5.1 Summary of Conclusions

In spite of its many remarkable advantages as a therapeutic agent for diabetes, GLP-1 is not immediately clinically applicable because of its extremely short half-life. The purpose of this research was to design the effective way of GLP-1 delivery for therapeutic use of GLP-1. GLP-1 is an insulinotropic hormone which increases insulin secretion in a glucose-dependent manner. Insulin secretion is only increased when concentrations of glucose is high and not at low to normal concentrations of glucose, therefore avoiding hypoglycemic episodes. The first half of the research studied the effects of GLP-1 delivery using a thermosensitive biodegradable triblock copolymer. The second half of this research was to design a new and effective GLP-1 delivery system for therapeutic use for type 2 diabetes treatment based upon construction of GLP-1 plasmid and its delivery.

To begin the study of the effect of GLP-1 delivery system using biodegradable injectable polymer, *in vitro* release experiment was performed to investigate the feasibility of GLP-1 polymeric drug delivery and to determine the duration of the system. The results from the experiment did show steady amount

of GLP-1 released from PLGA-PEG-PLGA triblock copolymer formulation depot more than ten days after single subcutaneous injection.

The animal experiment with the type 2 diabetic model performed for this research project also investigated the effects of polymeric delivery of GLP-1. It was evident that the GLP-1 released from the thermosensitive biodegradable hydrogel (PLGA-PEG-PLGA triblock copolymer) formulation is bioactive as it stimulates insulin secretion *in vivo*. Also, GLP-1 released from PLGA-PEG-PLGA triblock copolymer formulation depot results in the improved glucose tolerance. Therefore, it was concluded that it is feasible to use PLGA-PEG-PLGA triblock copolymer formulation with zinc-complexed GLP-1 as a 2 weeks delivery system, making it a twice-a-month injection depot.

For less frequent and more convenient administration of GLP-1, the GLP-1 gene delivery system was attempted in this study. GLP-1 plasmid delivery allowed sustained secretion of active GLP-1 for an extended time. Thus a major limitation of current GLP-1 therapy can be overcome. As GLP-1 stimulates the cells, more insulin production occurs in pancreatic β cells. Since no reactive hypoglycemia occurs even with higher concentrations of GLP-1, a potent promoter was used in this system. Normally, the GLP-1 is produced in intestinal L-cells, and matures through the posttranslational process. In the case of non-viral gene delivery, substantial amount of delivered DNA is taken up by hepatocytes. Thus, a furin cleavage site was placed in the GLP-1 coding region to release methionine that the start codon encodes. Therefore, the delivered GLP-1 plasmid/PEI complex could produce active GLP-1.

In conclusion, it has been established that GLP-1 gene delivery significantly decreased blood glucose concentration in a type 2 diabetic animal model. The results revealed that delivered GLP-1 gene stimulated insulin secretion, indicating that GLP-1 gene delivery represents a promising candidate for treatment of type 2 diabetes.

5.2 Further Research

GLP-1 is one of the most promising therapeutic agents for type 2 diabetes. It has been shown that GLP-1 administration can lead to a near-normalization of blood glucose levels in type 2 diabetic patients, even in patients with secondary failure of sulfonylurea and long-standing disease, admitted to hospital for insulin treatment [1, 2].

However, it has been shown that GLP-1 cannot be practically applied because of its extremely short half-life. Since the extremely short half-life is due to DPP-IV activity, DPP-IV resistant analogs have been developed as alternatives. The enzymatic cleavage occurs at the alanine in position two, thus analogs modified at this position were more resistant than GLP-1 itself. Such analogs retain a prolonged insulinotropic activity compared with native GLP-1. Therefore, GLP-1 dose can be reduced and its duration can be prolonged by using GLP-1 analog instead of native GLP-1. The effect of this GLP-1 analog should be carefully studied for clinical applications, because the GLP-1 analog will likely result in formation of antibodies in the body.

The results of many clinical studies demonstrate that GLP-1 may result in marked metabolic improvements, improvements in pancreatic functions, insulin sensitivity, and body weight. However, one study has clearly shown that the most important requirement for any therapeutic agent for type 2 diabetes is the ability to prevent progressive β-cell deterioration [3]. It has been suggested that GLP-1 has tropic effects for β-cells, and also an ability to protect and preserve β-cells in rodent model [4, 5, 6]. At present, no data are available with respect to tropic effects of GLP-1 on human β-cells. Since the turnover of β-cells in humans is much slower than that of rodents [7], tropic effects in humans may appear only after prolonged treatment with GLP-1. Therefore, further studies regarding tropic effects on human β-cell would be required for clinical use of GLP-1 for type 2 diabetes.

In addition, the effect of combination therapy of GLP-1 with other treatments will be beneficial. It has been shown that combination therapy of GLP-1 with metformin has additive effects [8].

For GLP-1 gene delivery, PEI was used as a nonviral carrier. Since viral carriers are expensive to produce, difficult to quality control, potentially damaging to the cells they infect, and most importantly they may cause potent immune responses that limit their use, it is desirable to develop nonviral systems. Nonviral gene delivery vectors offer numerous advantages such as excellent safety profiles, ability of carrying large amounts of DNA, and easy quality control. However, the current non-viral gene delivery systems including PEI have shown low transfection efficiency. Therefore, further development of the non-viral carrier

which has low cytotoxicity could improve the gene delivery efficiency and allow for the system to be delivered repeatedly when it is required.

There are many obstacles still to overcome in the clinical use of GLP-1 delivery system, and researchers are working to overcome each obstacle. With the combined efforts from each of fields, a successful and convenient GLP-1 treatment will be possible in near future.

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